AMINO ACID NEUROTRANSMITTERS; MECHANISMS OF THEIR UPTAKE INTO SYNAPTIC VESICLES

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ELSE MARIE FYKSE

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In the present work it was shown that GABA and L-glutamate (later termed glutamate) were taken up by a Mg^{2+} and ATP dependent mechanism into synaptic vesicles isolated from rat brain. The vesicular uptake differed clearly from the synaptosomal and glial cell uptake, both with respect to Na^+, Mg^{2+} and ATP dependency. The uptake of glutamate and GABA was inhibited by similar, but not identical concentrations of different ionophores and by inhibitors of the Mg^{2+}-ATPase. The uptake of glutamate was dependent on the presence of low concentrations of Cl^- or Br^- in the incubation medium, whereas the uptake of GABA was not. In addition the uptake of glutamate was more potently inhibited by blockers of Cl^- exchange than the uptake of GABA. The results indicate involvement of a Cl^- exchanger in the uptake of glutamate. The regional distribution in the brain of the uptake of GABA and glutamate was found to be different. The substrate specificity of the uptake of GABA and glycine was similar, and the vesicular uptake of GABA and glycine was competitively inhibited by different structure analogues. These results support the concept that synaptic vesicles are important for storage of amino acids in the nerve terminal. The mechanisms of the uptake of glutamate and GABA are different, whereas the mechanisms of the uptake of GABA and glycine seems to be similar.

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PREFACE

The work presented in this thesis was carried out at the Norwegian Defence Research Establishment (NDRE), Division for Environmental Toxicology, Kjeller, in the period 1986–1990. It is a part of a research program in synaptic transmission in the nervous system.

I wish to express my graditude to Professor Frode Fonnum, Head of the Division for Environmental Toxicology, for his excellent supervision, inspiration, support and constructive criticism during this work. I also want to thank the Director of the NDRE, Dir Erik Klippenberg, for providing research facilities.

My thanks are also extended to Dr Herbert Stadler, then at the Max Planch Institute für Biophysichalische Chemie, Arbeitsgruppe Neurochemie, Gøttingen and Professor Victor P Whittaker, then head of that department, for introducing me to the techniques dealing with isolation of synaptic vesicles. I would also extend my thanks to Dr Hege Christensen for inspiration, collaboration and helpful discussions, to Ms Evy Grini Iversen and Ms Marita Ljønes for giving me excellent techniqual help, and to all my other colleagues at the NDRE, Division for Environmental Toxicology, for their help and support.

I am grateful to Dr Rolf Hovik, Department of Physiology and Biochemistry, Dental School, University of Oslo, for inspiring support and helpful discussions during this work.

Else Marie Fykse

Kjeller, August 1991

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1 OBJECT OF INVESTIGATION

The object of the present study was to throw light on the mechanisms by which the amino acid neurotransmitters are stored within the nerve terminal. Previous studies by Naito and Ueda (1983) have shown that glutamate is taken up by an isolated synaptic vesicle fraction. In the same study, they did not find any uptake of γ-aminobutyrate (GABA). It was therefore still an open question at the start of this investigation if the neurotransmitter GABA was stored and released from synaptic vesicles.

The investigation can be divided into three following parts. (1) Investigation of whether GABA is taken up into a synaptic vesicle fraction and if the vesicle uptake could be distinguished from the plasma membrane uptake. (2) Characterization of the *in vitro* uptake of GABA and comparison to the uptake of other neurotransmitters, in particular glutamate. (3) The specificity of the uptake of the transmitter amino acids into synaptic vesicles has been investigated by studying regional distribution and inhibition of uptake.

The papers of the present thesis are listed below, and will be referred to in the text by their Roman numerals.

Paper I
Fykse E M and Fonnum F (1988): Uptake of γ-aminobutyric acid by a synaptic vesicle fraction isolated from rat brain, J Neurochem 50, 1237–1242.

Paper II
Fykse E M, Christensen H and Fonnum F (1989) Comparison of the properties of γ-aminobutyric acid and L-glutamate uptake into synaptic vesicles isolated from rat brain, J Neurochem 52, 946–951.

Paper III
Fykse E M and Fonnum F (1991): Transport of γ-aminobutyrate and L-glutamate into synaptic vesicles: Effect of different inhibitors on the vesicular uptake of neurotransmitters and on the Mg^{2+}ATPase, Biochem J 276, 363–367.

Paper IV
Fykse E M and Fonnum F (1989): Regional distribution of γ-aminobutyrate and L-glutamate uptake into synaptic vesicles isolated from rat brain, Neurosci Lett 99, 300–304.

Paper V
Christensen H, Fykse E M and Fonnum F (1991): Inhibition of γ-aminobutyrate and glycine uptake into synaptic vesicles, Eur J Ph-Mo 207, 73–79.

2 INTRODUCTION

2.1 Historical background

The quantal release of transmitters and the identification of the ultrastructural and molecular compounds have stimulated research groups for several decades. In 1950 and 1952 Fatt and Katz showed that release of acetylcholine at the frog neuromuscular junction was quantal (Fatt and Katz, 1950; 1952). This implies that discrete packages of acetylcholine are released. The release of single packages of acetylcholine from nerve endings can be monitored as postsynaptic miniature endplate potentials (m.e.p.p.s.). At the same time, electronmicroscopy had been developed to the degree that synaptic structures could be visualized in detail. Nerve endings were found to contain a large number of small vesicles with a diameter of about 50 nm (Sjöstrand, 1953; De Robertis and Bennet, 1955).

Later, application of subcellular fractionation techniques permitted the isolation of nerve endings (Gray and Whittaker, 1962). It also became possible to isolate vesicles in a highly purified preparation (De Robertis et al, 1963; Whittaker et al, 1963, 1964), and to show that the vesicles contained acetylcholine. The purity of the preparation and particularly its content of the enzyme choline acetyltransferase (ChAT) (EC 2.3.1.6) was a controversy for several years (McCaman et al, 1965; Fonnum, 1967, 1968). Later studies on the electromotor nerve terminal from the electric organ of *Torpedo* have contributed greatly to the development of the vesicular field. The advantage of the *Torpedo* electric organ is that it is purely cholinergic. Synaptic vesicles isolated from the electric organ of *Torpedo* are larger than vesicles isolated from other nerve terminals, (Whittaker, 1984) 90 nm instead of 50 nm in diameter. The simplest explanation for quantal release of transmitters would be the secretion of multimolecular packets of acetylcholine due to extrusion of the vesicular contents into the synaptic cleft. This has been termed the vesicle hypothesis of neurotransmitter release. The vesicle hypothesis has gained wide acceptance as a general explanation of transmitter release (Zimmerman, 1979; Ceccarelli and Hurlbut, 1980). Monoamines are present in high concentration in synaptic vesicles isolated from central and peripheral neurons, and the vesicle hypothesis has also been confirmed for these neurotransmitters (Smith and Winkler, 1972).

Despite the great acceptance of the vesicle hypothesis, a mechanism of acetylcholine release from the cytosolic pool has been suggested (Dunant, 1986). In this study it was suggested that the transmitters stored in vesicles constitute a reserve pool. A protein termed mediatophore has been isolated from the plasma membrane of *Torpedo* electric organ synaptosomes. After insertion into artificial liposomes the mediatophore has been shown to mediate Ca^{2+} dependent release of acetylcholine (Israel et al, 1986). Recently, a protein subunit of the mediatophore has been identified as a component of the synaptic vesicle proton pumping ATPase (Pirman et al, 1990).

2.2 Structure and function of synaptic vesicles

Synaptic vesicles have been isolated both from the electric organ of *Torpedo*, mammalian brain and spinal cord, and from the myenteric plexus of the guinea pig ileum. A great deal of the knowledge concerning the structure and function of synaptic vesicles has appeared from studies of vesicles from the electric organ of *Torpedo*. The more limited data concerning the mammalian brain synaptic vesicles may partly be due to the heterogeneity of the brain synaptic vesicle fractions, which probably consist of subpopulations of vesicles, each specific for different neurotransmitters.

Cholinergic synaptic vesicles from the electric organ of *Torpedo* are not a homogeneous pool of vesicles of the same size and density. Three subpopulations of cholinergic synaptic vesicles from electric organ of *Torpedo* have been found; the VP_0-, VP_1- and VP_2-vesicles (Zimmerman and Whittaker, 1977). The VP_0-vesicles are transported from the perikaryon to the terminal with fast axonal transport (Kiene and Stadler, 1987; Stadler and Kiene, 1987). The VP_0-vesicles have a protein composition identical to that of the vesicles isolated from nerve terminals, but they do not contain acetylcholine and ATP. In the terminal they accumulate acetylcholine and ATP and become the VP_1-vesicles. On arrival of an action potential at the nerve terminal, the vesicles

undergo exocytosis. After release, the vesicle are recycled (Zimmerman and Denston, 1977a; Zimmerman and Whittaker, 1977), and then they reaccumulate acetylcholine and ATP. The pool of the recycling vesicles constitutes the VP_2-vesicles. The VP_1-subpopulation of vesicles constitute the resting and depot pool of vesicles. The VP_2-vesicles are smaller and denser than the VP_1-vesicles due to storage of a smaller amount of acetylcholine and ATP, and they are localized closer to the nerve terminal than the VP_1-vesicles (Zimmerman and Denston, 1977b; Giompress et al, 1981). The actively recycling VP_2-vesicles probably contain most of the newly synthesized acetylcholine and ATP (Zimmerman and Denston, 1977b; Zimmerman, 1978), and they are thought to be responsible for the preferential release of the newly synthesized transmitter (Suszkiw et al, 1978). Stimulation of the electric organ increases the proportion of recycling vesicles (VP_2-type) in the total population of synaptic vesicles (Zimmerman and Denston, 1977a, b).

All neurons in the mammalian peripheral and central nervous system contain one or more distinct population of vesicles. They differ in size, shape and electrondensity. Evidence collected from biochemical analysis of subcellular fractions, immunocytological examination and pharmacological experiments indicates that the small type of vesicles (45–55 nm) from adrenergic and cholinergic nerve endings contains neurotransmitter and ATP (Fried et al, 1981; Zimmerman, 1982; Whittaker, 1986), but is devoid of neuropeptides. In addition to small vesicles, the cholinergic and noradrenergic terminals (varicosities) contain large vesicles measuring 80 to 120 nm in diameter. Analysis of particles isolated from peripheral and central nervous systems indicate that the large vesicles are the main storage organelles for neuropeptides (von Euler, 1963; Lundberg et al, 1981, Floor et al, 1982, Klein et al, 1982, Fried et al, 1985, 1986). The physiological importance of these peptides probably varies with the tissue and animal species since there are great differences in number and consequently in the storage capacity of the large vesicle population (Klein and Thureson-Klein, 1984, Douglas et al, 1986). The large vesicles constitute about 5–10 % of all vesicles in the terminal (Klein and Lagerkrantz, 1982).

Recently, new information has been gained concerning the structure and function of small synaptic vesicles from a mammalian brain. Mammalian brain synaptic vesicles have now been purified sufficiently to make identification, purification and characterization of the vesicle proteins possible. Some proteins associated with mammalian brain vesicles will be discussed briefly. Figure 1 shows a model of a mammalian brain GABAergic synaptic vesicle.

Synapsin I

Synapsin I is one of the best characterized synaptic vesicle-associated proteins (for review see Nestler and Greengard, 1986). Synapsin I has been found to be concentrated in nerve terminals, and under conditions of low ionic strength Synapsin I was associated with synaptic vesicles during their isolation (Huttner et al, 1983). The protein has been purified, and represents about 6% of the total protein present in highly purified vesicles (Huttner et al, 1983). In structure, the protein is elongated and highly asymmetric. It contains a tail-region and a head-region. One serine residue can be phosphorylated at the head-region, and two at the tail-region, all by different protein kinases (Huttner and Greengard, 1979; Huttner et al, 1981). Phosphorylation of the tail-region has been shown to decrease the binding of synapsin I to the vesicles, and facilitate the release of neurotransmitters. Phosphorylation may also alter the binding of Synapsin I to cytoskeleton proteins (Nestler and Greengard, 1986).

Synaptophysin (p38)

Synaptophysin has been identified independently by three different groups (Bock et al, 1974; Jahn et al, 1985; Wiedenmann and Franke, 1985). The protein is an integral membrane protein. On the basis of analysis of the amino acid sequence it has been proposed that synaptophysin spans the vesicle membrane four times, with the amino and carboxy terminal located on the cytoplasmic surface (Südhof et al, 1987). The cytoplasmic domain binds Ca^{2+} and synaptophysin is reported to be the major Ca^{2+} binding protein of synaptic vesicles (Rehm et al, 1986). The cytoplasmic carboxy tail undergoes tyrosine phosphorylation by tyrosine kinases (Barnekow et al, 1990). The purified synaptophysin forms a hexameric structure and a voltage dependent ion channel when incorporated in planar lipid bilayers (Thomas et al, 1988), and it has been suggested that this protein might be involved in exocytosis of synaptic vesicles during neurotransmission.

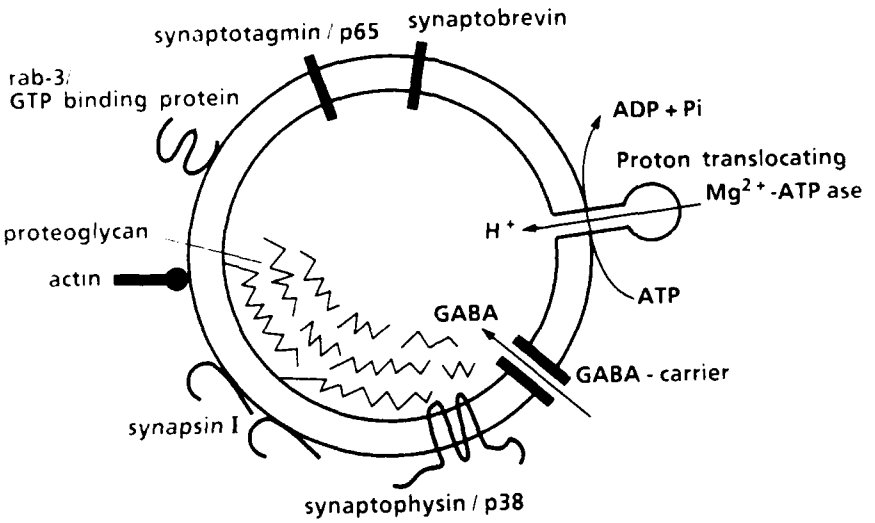

Figure 1 *A model of a mammalian brain GABAergic synaptic vesicle*

ATPase

There are three main classes of ATPases named P-, F-and V-type ATPases The plasma membrane (P-type) operates via a phosphoenzyme intermediate (e. g. Na^+/K^+- ATPase) (Forgac and Chin, 1985) In the plasma membrane they have a major role in maintaining the ionic homeostasis of the cell by controlled pumping of various cations across the cell membrane The eubacterial type (F-type) is present in eubacteria, mitochondria and chloroplasts. Their main functions is to phosphorylate ADP at the expense of a protonmotive force (Futai et al, 1989) The vacuolar proton (V type) ATPase is present in a variety of intracellular membrane bound organelles, including clathrin-coated vesicles (Forgac et al, 1983; Stone et al, 1983), endosomes (Galloway et al, 1983; Yamasihro et al, 1983), Golgi-derived vesicles (Glickman et al, 1983; Zhang and Schneider, 1983) and chromaffin granules The ATPase activity of chromaffin granules was discovered about three decades ago (Kirshner, 1962). Later evidence has clearly shown that the chromaffin granule ATPase is a proton pump responsible for generating the protonmotive force for catecholamine uptake (Bashford et al, 1976; Casey et al., 1976; Flatmark and Ingebretsen, 1977; Holz, 1978; Johnson et al, 1979). Whether all the vacuolar proton pumps in a cell are identical or belong to a family of closely related proteins, and how the mechanisms by which the activities of these pumps are regulated, are crucial but unanswered questions. One of the subunits, a proteolipid of 16 kDa, has been identified as a part of the proton channel (Sun et al, 1987). It has some sequence homology to the proton channel of the mitochondrial ATPase (Mandel et al, 1988), and they are thought to share a common evolutionary origin. The ATPases are often characterized on the basis

of their sensitivity to inhibitors, and the vacuolar ATPase is highly sensitive to N-etylmaleimide (NEM), an alkylating agent. It is insensitive to inhibitors of the plasma membrane ATPase, such as vanadate and ouabain, and inhibitors of the mitochondrial ATPases, such as oligomycin and azide (Pedersen and Cerafoli, 1987).

The Mg^{2+} activated H^+-ATPase of synaptic vesicles generates a proton electrochemical gradient (Stadler and Tsukita, 1984; Cidon and Sihra, 1989) The ATPase is required for uptake of neurotransmitters into synaptic vesicles. This will be discussed in more detail in the general discussion. Recently, it has become clear that the vesicular Mg^{2+}-ATPase belongs to the class of vacuolar ATPases (Maycox et al, 1988; Cidon and Sihra, 1989; Floor et al, 1990; Moriyama et al, 1990) The vacuolar H^+-ATPase of chromaffin granules is a multimeric protein composed of eight different subunits (Moriyama and Nelson, 1989; Nelson, 1991). The protein is composed of two distinct structures; a peripheral catalytic sector and a hydrofobic membrane sector. The vesicle H^+-ATPase is shown to be immunologically related to the chromaffin granule enzyme (Cidon and Sihra, 1989). A vanadate sensitive ATPase of the P-type has also been found and purified from the electric organ of *Torpedo* (Yamagata et al, 1989; Yamagata and Parsons, 1989). The function of this ATPase is unknown. This means that the cholinergic vesicles contain both a P-type ATPase and a V-type ATPase A vanadate sensitive ATPase has also recently been purified from chromaffin granule membranes (Moriyama and Nelson, 1988).

2.3 Storing of catecholamines and acetylcholine in chromaffin granules and synaptic vesicles

Since most workers agree that evoked release of acetylcholine and catecholamines occur by exocytosis of synaptic vesicles, the storage of neurotransmitters by vesicles is probably a critical and obligatory step in normal function of the nerves. The function of chromaffin granules is to store catecholamines in high concentration and, upon stimulation of chromaffin cells, to deliver the catecholamines into the extracellular space. The active uptake of catecholamines is driven by ATP hydrolysis. The activity of the vacuolar ATPase, builds up a proton electrochemical gradient which is the driving force for the uptake (for review see Njus et al, 1981) The accumulation of these substrates is sensitive to reserpine. Reserpine which is an alkaloid derived from the root of *Rauwolfia serpentina* competitively inhibits the uptake of catecholamines (Kirshner, 1962; Jonasson et al, 1964). The chromaffin cells have been used as a model system for studying uptake and release processes in brain vesicles. The uptake of catecholamines into brain vesicles has also been found to be driven by the proton motive force generated by a H^+-ATPase (Philippu and Beyer, 1973, Toll and Howard, 1978)

Progress in the study of acetylcholine storage in synaptic vesicles has been obtained by using pure vesicles isolated from the electric organ of *Torpedo*. These vesicles have an active transport system for acetylcholine (see Parsons et al, 1987). The uptake of acetylcholine is stimulated by Mg^{2+} ions and ATP and is inhibited by certain inhibitors of energy metabolism (Anderson et al, 1982). Uncouplers dissipate the proton electrochemical gradient that has been generated. Thus active uptake of acetylcholine is driven by a proton electrochemical gradient generated by the vesicular ATPase. In contrast to the uptake of catecholamines, the uptake of acetylcholine is stimulated by low concentrations of HCO_3^- (Koeningsberger and Parsons, 1980; Parsons and Koeningsberger, 1980) The active uptake of acetylcholine is inhibited noncompetitively by l-trans-2-(4-phenylpiperidino)-cyclohexanol (vesamicol, formerly AH5183) (Anderson et al, 1983). Vesamicol was originally discovered as a neuromuscular blocking agent (Marshall, 1970).

2.4 Storing of amino acids in synaptic vesicles

It is generally accepted that the amino acids GABA and L-glutamate are major neurotransmitters in the mammalian central nervous system (Krnjevic, 1970; Fonnum, 1984). GABA and glutamate are quantitatively the most important neurotransmitters in the mammalian central nervous system. Glutamate is present in at least four different pools in the brain: As transmitter in glutamatergic terminals, as precursor for GABA in GABAergic terminals, as a metabolic

component in other neuronal structures and in glial cells. This has greatly complicated the analysis of the releasable amino acid transmitter pool.

The use of subcellular fractionation technique to identify the different pools of glutamate has until now not been successful. In 1989 Burger and coworkers reported that glutamate was enriched in the vesicle fraction. Earlier, several workers have failed to do this. Burger and coworkers (1989) used a rapid isolation procedure for isolation of synaptic vesicles based on immunoisolation. The storage seems to be labile, requiring the preservation of an energy gradient across the vesicle membrane. It is shown by Carlson and Ueda (1990) that the existence of an electrochemical proton gradient across the vesicular membrane is required in order to maintain steady-state levels of glutamate accumulated by a vesicle fraction *in vitro*, but still there is some efflux. Treatment of the vesicles by NEM blocks some of this glutamate efflux (Carlson and Ueda, 1990). NEM also prevents efflux of endogenous glutamate from a vesicle fraction (Burger et al, 1989). Morphological studies by Storm-Mathisen and coworkers (1983) led to the first visualization of GABA and glutamate in neurons by immunocytochemistry.

So far vesicle specific transport activities have been described for the amino acids glutamate (Paper II; Disbrow et al, 1982; Naito and Ueda, 1983, 1985; Maycox et al, 1988), GABA (Paper I; Hell et al, 1988; Kish et al, 1989), and glycine (Kish et al, 1989; Christensen et al, 1990). It is apparent that all the uptake carriers are active transporters dependent upon the proton electrochemical gradient. No specific inhibitors, such as reserpine and vesamicol in the case of catecholamine and acetylcholine uptake, are found for the uptake of amino acids. However, the uptake of glutamate is competitively inhibited by a peptide containing halogenated ergot bromocriptine (Carlson et al, 1989a). The uptake of GABA and glycine is competitively inhibited by structure analogues (Paper V). It is also reported that a nerve terminal cytosolic factor inhibits the ATP dependent vesicular uptake of glutamate in a dose dependent manner (Lobur et al, 1990). The endogenous factor may have a function in regulation of the transmitter pool of glutamate.

The ontogeny of the vesicular uptake of glutamate, GABA and glycine has been investigated in brain and spinal cord. The increase in vesicular uptake activity parallels synaptogenesis (Kish et al, 1989; Christensen and Fonnum, 1991a,b). This indicates the importance of synaptic vesicles in amino acid neurotransmission. The ontogeny of the high affinity uptake of glutamate over plasma membranes has been shown to increase with the time course similar to that of the vesicular uptake. In contrast, the developmental time course of the uptake of GABA is different (Christensen and Fonnum, 1991b). The plasma membrane uptake of GABA is found to have a distinct maximum during the second postnatal week (Schousboe et al, 1976). Functional reconstitution of carriers in proteoliposomes may provide insight into energetic and mechanistic aspects of the transport cycle. The carriers for the uptake of glutamate (Maycox et al, 1988; Carlson et al, 1989b) and GABA (Hell et al, 1990) have been reconstituted in proteoliposomes.

During the last few years, new evidence has appeared which shows that synaptic vesicles are important for storage and exocytotic release of amino acids. The fact that amino acids are actively accumulated into synaptic vesicles *in vitro* strongly supports the validity of the vesicle hypothesis for amino acids.

3 DISCUSSION OF METHODS

3.1 Purification of synaptic vesicles

The present study deals with the uptake of amino acid neurotransmitters into synaptic vesicles isolated from rat brain. Different methods have been used for the isolation of synaptic vesicles, and the original method described by Whittaker and coworkers (1964) has been used in the present study. Synaptic vesicles were isolated from a crude synaptosomal fraction subjected to hypo-osmotic lysis to release the vesicles, and the vesicles were further purified by sucrose density gradient centrifugation. The different fractions were tested for their GABA and glutamate uptake activity. The highest specific uptake activities were due to vesicles floating in 0.4 M sucrose, but 0.6 M sucrose also contained uptake activities. This is in agreement with the distribution of organelles in a sucrose gradient described by Whittaker and coworkers (1964). At the interface between 0.4 M and 0.6 M sucrose and in 0.6 M sucrose they found some synaptic vesicles, often in clumps, microsomes and occasional myelin fragments.

Another method for isolation of synaptic vesicles was described by De Robertis et al (1963). This method is based on osmotic shock of a crude synaptosomal fraction, followed by differential centrifugation into three subfractions, M_1, M_2, and M_3. In subfraction M_1 myelin fragments and membrane structures are accumulated. The major part of the high speed centrifugation pellet M_2 consists of synaptic vesicles, but this fraction is found to be more contaminated by microsomes and membrane structures than the one obtained by Whittaker et al (1964). M_3 is the final supernatant or soluble subfraction. A modification of this method has been applied due to the small amount of material obtained from the different brain structures (Paper IV). Myelin and microsomes were separated from the synaptosomal fraction by a sucrose density gradient. This gives a vesicle fraction less contaminated by membranes than the vesicular fraction obtained by De Robertis et al (1963).

Further purification of synaptic vesicles has been performed by different methods. These methods will be discussed in light of the uptake function of the vesicle fractions. Naito and Ueda (1983) isolated a vesicle fraction from bovine brain by osmotic shock of a synaptosome fraction, sucrose gradient and immunprecipitation with anti-synapsin I, but they did not found any uptake of GABA. Later they described an uptake of GABA into a vesicle fraction isolated from rat cerebrum. This vesicle fraction was isolated by lysis of a crude synaptosome fraction and centrifugation in a Percoll gradient. They obtained a GABA/glutamate uptake ratio of 0.03 (Kish et al, 1989). In contrast, in the present work a GABA/glutamate uptake ratio of about 0.25 is found (Papers II, III, IV, V). The reason for this discrepancy may be that Ueda and coworkers are destroying their vesicles during the isolation procedure. Some neuroanatomical studies have reported that the GABAergic and glycinergic vesicles are elliptic in shape (Bodian, 1972), which may imply that these vesicles are more labile than the glutamate vesicles. Thus, several procedures may lead to partly destruction of the GABA uptake activity. Isolation of synaptic vesicles have also been performed in a Nycodenz gradient (Floor et al, 1988). In contrast to the sucrose gradient, the osmolarity of Nycodenz and Percoll gradients can be kept constant over a wide range of densities. Synaptic vesicles are banded in 0.4 M sucrose which is close to iso- osmolarity. Therefore, the constant osmolarity of Nycodenz and Percoll is more important for denser organelles such as synaptosomes. Synaptosomes are banded between 0.8 M and 1.0 M sucrose.

In Paper III, the vesicle fraction was further purified on a controlled pore glass column. The specific activity of the uptake of GABA and glutamate was doubled. Due to the low capacity and low increase of the specific uptake activities, the vesicle fraction was usually not isolated by gel filtration. The synaptic vesicles obtained by hypo-osmotic lysis of synaptosomes and sucrose gradient centrifugation have so far shown the highest GABA/glutamate uptake ratio. The ratio of about 0.25 is in agreement with the ratio between synaptosomal GABA and glutamate uptake obtained by Christensen and Fonnum (1991c). Hell et al (1988) have isolated synaptic vesicles by sucrose gradient and gel filtration on a controlled pore glass column from brain tissue frozen in liquid nitrogen. In liquid nitrogen the nerve terminals are effectively broken up and direct preparation and isolation of synaptic vesicles is possible (Whittaker et al., 1972; Tashiro and Stadler, 1978). Hell et al (1988) obtained a ratio between the uptake of GABA and glutamate of about 0.14. In agreement with the results of Paper III, the specific activity of the uptake of GABA

and glutamate was doubled when the vesicle fraction was chromatographed on a controlled pore glass column. Synaptic vesicles further purified by gel filtration after gradient centrifugation are less contaminated by microsomes and other membrane structures, but physiological function beyond purity of synaptic vesicles seems to be important when it comes to uptake studies.

Due to the large amount of Mg^{2+} activated ATPase in all membranes, the vesicular ATPase was measured in a highly purified vesicle fraction. The activity of the Mg^{2+}- ATPase (Paper III) was distributed in two peaks when synaptic vesicles isolated by sucrose gradient were further purified on a controlled pore glass column. The activity of acetylcholine esterase (AChE) (EC 3.1.1.7), a marker enzyme for plasma membranes, coeluted with the first of these two peaks, and most of the uptake activity coeluted with the second peak of Mg^{2+}-ATPase. A small part of the uptake activities (less than 5 %) coeluted with the membrane fraction Most likely, some synaptic vesicles, or aggregated vesicles coeluted with the membrane fraction.

The high affinity plasma membrane uptake of neurotransmitters is dependent on the Na^+ gradient across the plasma membrane (for review see Kanner and Schuldiner, 1987; Fonnum et al, 1980). The vesicular GABA and glutamate uptake is not stimulated by Na^+ ions (Paper I; Naito and Ueda, 1983). When the synaptosomal and vesicular uptake of GABA were performed under identical conditions only the synaptosomal uptake was highly stimulated by addition of 50 mM NaCl (almost 15 fold). A low concentration of GABA (44 µM) was used, due to the higher affinity of the plasma membrane uptake. The uptake of GABA into synaptosomes was not reduced by removal of ATP and Mg^{2+} (Paper I). Therefore, contamination in the vesicular fraction by plasma membranes cannot be of any significance for the vesicular uptake. The present results also show that the vesicular uptake is dependent on ATP, Mg^{2+} and an intact electrochemical proton gradient across the vesicle membrane (Papers I, II).

3.2 Blank values

The vesicular uptake measured could not be due to binding of substrate to membranes. The vesicles bound to the filters during the uptake procedure were osmotically sensitive. The inhibitory effect of the proton ionophores also indicates uptake instead of binding (Paper II). In the present study, the blank values have been treated in the same way as the samples, but they were incubated at 0°C instead of 30°C. At 30°C the uptake is maximal. For the uptake of glutamate, the blank values constitute about 10–15 % of the radioactivity retained on the filters, and for GABA 20–25 %. Most of this is, however, binding of substrate to the filters (70 %). The blank values did not vary when different test agents were added as well. Other groups have used the activity at 30°C in the absence of ATP as blank values (Kish et al, 1989; Hell et al, 1990). In the absence of ATP the uptake of GABA and glutamate is reduced by 80–90 % (Paper I; Naito and Ueda, 1985). The activity measured in the absence of ATP is not necessarily due to binding, at least not in our vesicle fraction. Endogenous ATP in the vesicle fraction, may be responsible for uptake activity in the absence of exogenous ATP. Therefore, the blank values were incubated at 0°C, but otherwise treated in the same way as the samples.

3.3 Kinetic conditions

The Km value for the uptake of GABA has been determined to be 5.6 mM (Paper I). Later Kish et al. (1989) obtained nearly the same value. For glutamate uptake, the Km value has been determined to about 1 mM (Naito and Ueda, 1985; Maycox et al., 1988). The experiments in Paper I were performed with a low concentration of GABA (44 µM). Later the substrate concentration was increased due to the kinetic properties of the system. It is more correct to use a low mM concentration of the substrate than a low µmolar concentration, and specially in experiments where kinetic conditions are studied. Ideally the substrate concentration should be of the order of at least the Km value, but the specific radioactivity would be to low to permit uptake measurement. As a compromise, a concentration of 1 mM was used (Papers II, III, IV, V). The samples were also incubated for 1.5 or 3 minutes. The system is not saturated at 3 minutes, therefore the rate of the uptake was measured.

The uptake of GABA and glycine in brain and spinal cord vesicles have been studied, and inhibition of the GABA uptake by glycine and *vice versa* is reported. One may conclude from these studies that the specificity of the uptake of GABA and glycine is similar (Paper V). This is in contrast to the results of Kish et al (1989), who concluded that the uptake of GABA and glycine are different. They did not find any inhibition of the uptake of GABA by glycine or *vice versa*. The reason for this discrepancy may be that Kish et al (1989) used inadequate kinetic conditions. They used a substrate concentration of 150 μM, which is far below the Km-values for the uptake systems, and 10 minutes incubation time. At 10 minutes the uptake of GABA and glycine is saturated.

4 GENERAL DISCUSSION

In the present study I provide evidence for a Mg^{2+} and ATP dependent in vitro uptake of GABA into synaptic vesicles. Knowledge of the mechanisms of vesicular uptake of the inhibitory neurotransmitters GABA and glycine and the excitatory neurotransmitter glutamate is essential for the understanding of the transmitter function of the different amino acids. The transport systems have been studied in detail both with regard to kinetics, inhibitors, regional distribution and specificity. On the basis of the present investigation, this discussion will focus on the following points: Comparison of the mechanisms of uptake of GABA and glutamate, with emphasis on the different effects of anions (4.1). The specificity of the uptake of GABA, glycine and glutamate, and the regional distribution of the uptake of these amino acids in the brain (4.2).

4.1 Comparison of the mechanisms of uptake of GABA and glutamate

The kinetic properties, energy demand, specificity and inorganic ion requirements of the vesicular and granular transport processes are different from that observed in the plasma membrane and mitochondrial membrane. One function of the neurotransmitter transport across the plasma membrane is to terminate the overall process of synaptic transmission. The different properties of the synaptosomal uptake and the vesicular uptake of GABA have been compared (Paper I). The main difference is that the plasma membrane uptake is dependent on Na^+, whereas the vesicular uptake is not. The GABA and glutamate carriers in the vesicle membrane have lower affinities than the plasma membrane carriers (Paper I; Fonnum et al, 1980; Naito and Ueda, 1985; Kish et al, 1989). The vesicular uptake is stimulated by ATP and Mg^{2+}, whereas the high affinity plasma membrane uptake is not. Christensen and coworkers (1990) have shown that also a low affinity plasma membrane uptake of glycine is stimulated by Na^+. This uptake is not dependent on ATP and Mg^{2+}, and it is not inhibited by the proton ionophore carbonyl cyanide m-chloro phenylhydrazone (CCCP). This clearly demonstrates the difference between the vesicular low affinity uptake, and the high affinity and the low affinity plasma membrane uptake.

Vesicular uptake of GABA and glutamate is found to be inhibited almost to the same extent by the proton ionophore CCCP (Paper II). Different groups have reported different effect of the ionophore nigericin on the uptake of glutamate (Paper II; Naito and Ueda, 1985; Cidon and Sihra, 1989; Moriyama et al, 1990). Nigericin induces an exchange of H^+/K^+ across a membrane in the presence of K^+ ions. I (Paper II), in conformity with Naito and Ueda (1985), report a potent inhibitory effect of nigericin on the uptake of glutamate in the presence of K^+. In contrast, in two other Papers no inhibitory effect of nigericin was observed (Cidon and Sihra, 1989; Moriyama et al, 1990). *In the latter works a much lower concentrations of K^+ (4–10 mM) was used. This may* explain the discrepancy between the results.

Glutamate accumulation in vesicles is dependent on a membrane potential gradient across the vesicle membrane (Maycox et al, 1988; Cidon and Sihra, 1989; Shioi et al, 1989; Moriyama et al, 1990). A vacuolar proton ATPase generates a membrane potential (positive inside), a proton gradient or both. The ATPase generates a large proton gradient in the presence of a high concentration of Cl. In the absence of permeant anions in the vesicular fraction the membrane potential generated is maximal (Maycox et al, 1988). It has been shown that dissipation of the pH component does not affect the glutamate uptake, and that the uptake is maximal where the membrane potential is maximised. Therefore, the uptake is solely dependent on the electrical gradient generated by the ATPase (Maycox et al, 1988). The positive membrane potential across the vesicle membrane is only slightly reduced during the uptake of glutamate (Maycox et al, 1988). Thus charge balance is largely maintained during net accumulation. At neutral pH, glutamate is anionic, so that compensation of inward cationic fluxes or outward anionic fluxes probably is associated with uptake. Maximal uptake of glutamate occurred at a concentration of about 4–10 mM Cl (Papers II, III; Naito and Ueda, 1985), which is in the same range as the physiological intracellular concentration. The reduced uptake of glutamate in the absence of Cl probably reflects a direct involvement of Cl in the process. An alternative model involving a H^+/glutamate antiport has been postulated (Shioi and Ueda, 1990). The intravesicular Cl itself or a Cl efflux may enhance the presumed H^+/glutamate antiport. Zwitterionic glutamate molecules are supposed to be taken up by the vesicles. Transported glutamate will dissociate and liberate H^+-

ions inside the vesicles, thus facilitating a further influx of glutamate. As demonstrated by Maycox et al (1988) acidification of the vesicles will inhibit the transport of glutamate. Removal of the H^+-ions by a H^+/Cl^- symport may be necessary for the glutamate uptake. The uptake of catecholamines is shown to be maximal in the presence of both a membrane potential and a proton gradient (Holtz, 1978; Johnson et al, 1979).

Hell et al (1990) concluded that the uptake of GABA is driven both by the proton gradient and by the membrane potential. The uptake of GABA is not stimulated by low concentrations of Cl^- or Br^- (Papers II, III). This is in agreement with Kish et al (1989). In contrast, Hell et al (1990) found that the uptake of GABA was reduced by 40% in the absence of exogenous Cl^-. Maximal uptake of GABA occurred in the range of 4–50 mM Cl^-. Endogenous Cl^- in the vesicle fraction may be responsible for the uptake of GABA in the absence of exogenous Cl^-, but this is not very likely. In contrast, even 1 mM Cl^- or Br^- stimulated the uptake of glutamate 3 and 4 fold, respectively. In a synaptic vesicle fraction isolated by a controlled pore glass column (Paper III), the uptake of GABA was not stimulated by Cl^- ions (results not shown). However, it is possible that the ATPase of GABAergic vesicles uses efflux of a cation to generate a proton gradient across the vesicle membrane. Further investigation is needed to be able to confirm this statement.

The stilbene disulfonate derivates SITS (4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid) and DIDS (4,4'-diisothiocyano-2,2'-stilbene-disulfonic acid) are known to be blockers of anion-exchange in erythrocytes. The site of action in erythrocytes is the protein Band 3, and the Cl^-/HCO_3^- exchange is inhibited (Cabantchick et al, 1978). 5-Nitro-2-(3-phenylpropylamino)-benzoic acid (N144) is a more specific anion channel antagonist when tested in kidneys (Wangemann et al, 1986). The uptake of glutamate is inhibited more potently by SITS, DIDS and N144 than the uptake of GABA. This is consistent with the fact that glutamate uptake is highly stimulated by low concentrations of Cl^- or Br^- (Papers II, III; Naito and Ueda, 1985). In chromaffin granules SITS inhibited the Cl^- stimulated Mg^{2+}ATPase activity, and the inhibition was competitive with respect to Cl^- ions (Pazoles et al, 1980). SITS also inhibited accumulation of $^{36}Cl^-$ by chromaffin granules (Pazoles and Pollard, 1978). The stilbene disulfonate derivates are also known to block proton transport. The proton pump activity in a subfraction of rat liver highly enriched in uncoated endocytotic vesicles was totally inhibited by 25 μM SITS. The IC_{50} value was determined to 3.5 μM (Flatmark et al, 1985). The inhibition of the proton transport by SITS did not affect the overall Mg^{2+}-ATPase activity of that system (Flatmark et al, 1985). The proton uptake activity in chromaffin granules is also found to be much more sensitive to anions than the ATPase activity (Moriyama and Nelson, 1987). SITS and N144 inhibited the vesicular Mg^{2+}-ATPase activity to a low extent compared to the effect on the vesicular uptake (Paper III). The vesicular H^+-ATPase belongs to the class of vacuolar enzymes (Cidon and Sihra, 1989; Floor et al, 1990), and the Mg^{2+}-ATPase of the glutamatergic and GABAergic vesicles are probably similar. As mentioned earlier, the uptake of glutamate is shown to be driven by the electrical potential, which is maximal in the absence of permeant anions (Maycox et al, 1988). It is therefore reasonable that the more potent effect of SITS, DIDS and N144 on glutamate uptake is due to an effect on the glutamate carrier. The uptake of GABA was not stimulated by anions, and is inhibited to a less extent by SITS, DIDS and N144 (Paper III). This implies that no anion related site is involved in the uptake of GABA. Recently, Maycox et al (1990) provided evidence for functional separation of the ATPase and transmitter uptake activity. They reported that the proton pump of bacteriorhodopsin can substitute for the endogenous proton pump of synaptic vesicles. The uptake of glutamate was strongly reduced when the concentration of Cl^- was reduced. Thus the glutamate carrier seems to be dependent on Cl^-, and a glutamate/Cl^- antiport would be a reasonable explanation. However, more evidence is needed to support this view.

4.2 Specificity and regional distribution of the uptake of GABA, glycine and glutamate

The distribution of the vesicular uptake of GABA and glutamate in different brain regions is different (Paper IV). This is in agreement with the fact that the enzyme synthesizing GABA, glutamate decarboxylase (EC 4.1.1.15), is localized in specific GABAergic nerve terminals (Fonnum et al, 1970). The subcortical telencephalon, which contains among others the regions hypothalamus, globus pallidus and substantia nigra, showed the highest vesicular uptake of GABA (Paper IV). These regions are known to be rich in GABAergic terminals (Ottersen and

Storm-Mathisen, 1984; Fonnum, 1987). The cerebellar granule cells are considered to be glutamatergic (Hackett et al, 1979), and the Purkinje cells are GABAergic neurons (Fonnum et al, 1970). Uptake of glutamate has been studied in a synaptic vesicle fraction isolated from cerebellar mutant mice. The uptake of glutamate was reduced by 60 % in vesicles from mice lacking granule cells, but not in vesicles from mice lacking Purkinje cells (Fischer-Bovenkerk et al, 1988). The high affinity uptake of GABA and glutamate is also differently distributed (Paper IV; Fonnum et al, 1980). Therefore, the glutamatergic and GABAergic nerve terminals seem to differentiate between glutamate and GABA on three levels, namely the high affinity uptake, the distribution of glutamate decarboxylase, and the vesicular uptake.

Christensen and Fonnum (1991c) have found that the ratio between the vesicular uptake of GABA and glycine is similar in cerebral cortex, subcortical telencephalon, whole brain, and spinal cord. This is not in agreement with the expected distribution of glycinergic neurons. Glycine is proposed to be an inhibitory neurotransmitter in the interneurons of spinal cord and in medulla (Johnston and Iversen, 1971). The supraspinal distribution of vesicular glycine uptake is probably due to uptake into non-glycinergic vesicles. The results of Paper V, that the uptake of glycine is competitively inhibited by GABA and *vice versa* support the idea that glycine is taken up into non-glycinergic neurons in supraspinal regions. In addition, the structure analogues GABA, glycine and β-alanine are taken up into synaptic vesicles isolated from rat brain and rat spinal cord (Paper V). The high affinity uptake of GABA and glycine are different (Balcar and Johnston, 1973). This means that the plasma membrane of GABAergic terminals transport GABA, and the plasma membrane of glycinergic terminals transport glycine. The concentration of GABA in GABAergic terminals has been estimated to be 50–150 mM (Fonnum and Wahlberg, 1973). It is therefore reasonable to expect a great difference in the concentration between GABA and glycine in GABAergic neurons and the vesicles will predominantly accumulate GABA. In addition, GABA has higher affinity for the vesicular transporter than glycine (Paper I; Kish et al, 1989;Christensen et al, 1990). In this way nature seems to be able to cope with the fact that the specificity of the vesicular GABA and glycine transporter is similar.

There has been some dispute concerning the results on the specificity of the vesicular GABA and glycine transporters. As pointed out earlier (discussion of methods), Kish et al (1989) obtained a low ratio of GABA/glutamate uptake (0.03) and glycine/GABA uptake (0.13), and in an earlier study they did not find any uptake of GABA at all (Naito and Ueda, 1983). These results indicate that they have problems with isolating GABAergic vesicles. Therefore, to detect any vesicular glycine uptake can be difficult due to the lower affinity of the glycine uptake. In addition they did not find any inhibition of the uptake of GABA by glycine and *vice versa*. This was probably due to the different kinetic conditions that was used (Kish et al, 1989). They concluded that the properties of GABA and glycine uptake are different, and that GABA and glycine are taken up into different vesicle populations. The specificity of the glycine uptake will be further discussed elsewhere (Christensen Dr Scient thesis 1991).

The findings that GABA and glycine can be taken up into the same vesicle population are interesting, in view of the colocalization of GABA and glycine immunoreactivity in cerebellum (Ottersen et al, 1988), cochlear nuclei (Wenthold, 1987) and retina (Yazulla and Yang, 1988). It has also been suggested by Ottersen et al (1990) that GABA and glycine may be released from the same neuron, at least from the cerebellar Golgi cell terminals.

It should be kept in mind that the uptake of noradrenaline and dopamine in synaptic vesicles prepared from rat brain is relatively non-specific. Noradrenaline containing vesicles can take up noradrenaline, dopamine and serotonin. In vesicle fractions from whole brain dopaminergic vesicles are responsible for a significant portion of the noradrenaline uptake (Slotkin et al, 1978). It is also shown that the vesicles isolated from corpus striatum exhibited the same ratio of uptake of dopamine/noradrenaline as did vesicles from cerebral cortex. Noradrenaline also competitively inhibited the dopamine uptake (Slotkin et al, 1978). In addition, both dopaminergic and noradrenergic nerve endings in the brain can take up either catecholamine (Snyder et al, 1970), but the regional distribution of these neurotransmitters in the brain is different, e. g. corpus striatum contains large quantities of dopamine with very little noradrenaline (Moore and Bloom, 1978, 1979).

In general, the vesicular uptake of GABA, glycine and catecholamines is non-specific. This non-specificity turns out to be a rule rather than an exception in nature. In contrast, the transporter of the glutamatergic vesicles seems to be specific for glutamate (Paper IV; Fischer-Bovenkerk, 1988). The Na^+ dependent glutamate uptake system in nerve endings does not distinguish between glutamate and aspartate (Logan and Snyder, 1972; Davies and Johnson, 1976). Aspartate, suggested to be an excitatory neurotransmitter in a few pathways in the central nervous system, is not taken up into the vesicles (Paper V; Naito and Ueda, 1983). So far investigated, glutamate is the only neurotransmitter which has a vesicular carrier stimulated by a low concentration of Cl. A glutamate/Cl antiport or a glutamate carrier coupled to a Cl channel may be involved in the uptake of glutamate.

5 CONCLUSIONS

1) The inhibitory neurotransmitter GABA is taken up into mammalian synaptic vesicles (Paper I). Both the uptake of GABA and glutamate are driven by an electrochemical gradient generated by a Mg^{2+}-ATPase (Paper II).

2) The uptake of glutamate is stimulated by low concentrations of Cl or Br, while the uptake of GABA is hardly affected. The uptake of glutamate is more potently inhibited by blockers of anion exchangers than the uptake of GABA. A possible mechanism for the uptake of glutamate may be a glutamate/Cl antiport (Paper III).

3) The specificity of the uptake of GABA and glutamate is different, and the transmitters are taken up into different populations of synaptic vesicles. The substrate specificity of the uptake of GABA and glycine is similar, and both are taken up into brain vesicles and spinal cord vesicles *in vitro* (Papers IV, V). Thus the vesicular uptake does not differentiate between GABA and glycine as transmitter candidates in specific terminals.

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PAPER I

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Uptake of γ-Aminobutyric Acid by a Synaptic Vesicle Fraction Isolated from Rat Brain

Else M. Fykse and Frode Fonnum

Division for Environmental Toxicology, Norwegian Defence Research Establishment, Kjeller, Norway

Abstract: γ-Aminobutyric acid (GABA) was taken up by a MgATP-dependent mechanism into synaptic vesicles isolated by hypoosmotic shock and density gradient centrifugation. The properties of the vesicular uptake differed clearly from those of synaptosomal and glial uptake, both with respect to Na^+, Mg^{2+}, and ATP dependence and with respect to response to general GABA uptake inhibitors such as nipecotic acid, diaminobutyric acid, and β-alanine. The uptake showed a K_m of 5.6 m*M* and a net uptake rate of 1,500 pmol/min/mg of protein. It is suggested that the vesicular uptake of GABA is driven by an electrochemical proton gradient generated by a Mg^{2+}-ATPase. **Key Words:** Synaptic vesicles—Synaptosomes—γ-Aminobutyric acid—γ-Aminobutyric acid uptake. **Fykse E. M. and Fonnum F.** Uptake of γ-aminobutyric acid by a synaptic vesicle fraction isolated from rat brain. *J. Neurochem.* **50**, 1237–1242 (1988).

γ-Aminobutyric acid (GABA) is probably the major inhibitory neurotransmitter in the CNS (Krnjević, 1970; Fonnum, 1978, 1987). It is well established that glutamic acid decarboxylase (EC 4.1.1.15), the enzyme that synthesizes GABA, is highly localized in the nerve terminal, probably in the cytosol (Salganicoff and De Robertis, 1965; Fonnum, 1968). Attempts to show an enrichment of GABA in synaptosomes or synaptic vesicles compared with other subcellular fractions have not been very convincing (De Belleroche and Bradford, 1973; Lahdesmäki et al., 1977; Wood and Kurylo, 1984). In fact, it was earlier concluded that vesicles do not contain amino acids in any significant concentration (Mangan and Whittaker, 1966; Rassin, 1972; Kontro et al., 1980). The lack of evidence for an enrichment of GABA in vesicles has been attributed to the possible leakage of the amino acids during the subcellular fractionation procedure.

Recent evidence indicates that L-glutamate is taken up in an ATP-dependent manner by synaptic vesicles isolated from bovine brain by antibodies against protein I (Naito and Ueda, 1983, 1985). This supports the notion that synaptic vesicles may be involved in synaptic transmission of amino acids. Naito and Ueda (1982), however, failed to show uptake of GABA into the immunoprecipitated synaptic vesicle fraction. Recently, Orrego et al. (1986) also failed to show uptake of GABA into a vesicle fraction.

In the present work, we have, therefore, reinvestigated the uptake of GABA into synaptic vesicles isolated from rat brain. We have also compared the vesicular and synaptosomal uptake under different conditions.

MATERIALS AND METHODS

GABA, ATP (disodium salt), carbonylcyanid-*m*-chlorophenylhydrazone (CCCP), ouabain, L-glutamate (disodium salt), D-aspartate, diaminobutyric acid (DABA), nipecotic acid, and β-alanine were purchased from Sigma Chemical Co. (U.S.A.). Oligomycin was obtained from Serva (GmbH). [2,3-^{3}H]GABA (71.5 Ci/mmol) was from Amersham (U.K.).

Purification of synaptosomes and synaptic vesicles

Male Wistar rats, weighing 200–250 g, were used in all experiments. Animals were killed by decapitation, and the brains were quickly removed. The subcellular fractionation was carried out according to the original procedure of

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Address correspondence and reprint requests to Dr. E. M. Fykse at Division for Environmental Toxicology, Norwegian Defence Research Establishment, P.O. Box 25, N-2007 Kjeller, Norway.

Abbreviations used: CCCP, carbonylcyanid-*m*-chlorophenylhydrazone; DABA, diaminobutyric acid; GABA, γ-aminobutyric acid.

Whittaker et al. (1964), except that 10 m*M* Tris-HCl (pH 7.4) and 1.0 m*M* EGTA were included in the sucrose solution (Stadler and Tsukita, 1984). The crude synaptosomal pellet (P_2) was osmotically shocked by resuspension in 0.1 m*M* EGTA and 10 m*M* Tris-HCl buffer (pH 7.4) and centrifuged at 17,000 *g* for 30 min. The remaining supernatant containing vesicles was subjected to sucrose density gradient centrifugation in a Contron TST 28.38 rotor at 65,000 *g* for 2 h, and the vesicle fraction (D) was isolated from 0.4 *M* sucrose as originally described. In some cases, the D fraction was diluted with 0.15 *M* KCl and recentrifuged at 100,000 *g* for 3 h, and the pellet was used in the uptake experiments.

A crude synaptosomal pellet (P_2) resuspended in 0.25 *M* sucrose and 5 m*M* Tris-HCl (pH 7.4) was subjected to GABA uptake experiments.

Assay for GABA uptake

GABA uptake was determined essentially as described by Naito and Ueda (1982, 1985) for vesicular glutamate. The standard incubation mixture for assaying vesicular and synaptosomal GABA uptake contained 0.25 *M* sucrose, 5 m*M* Tris-HCl (pH 7.4), and 4 m*M* $MgSO_4$. The standard incubation medium for synaptosomes contained, in addition, 50 m*M* NaCl. Synaptic vesicles (0.2–0.3 mg of protein) and synaptosomes (0.05 mg of protein) were preincubated in 275 μl of standard incubation mixture for 15 min at 30°C. [^{3}H]GABA (final concentration = 44 μ*M*; 0.1 Ci/mmol) alone or with ATP (final concentration = 2 m*M*; disodium salt neutralized with Tris base) was added in 25 μl, and the mixture was further incubated for 3 min at 30°C. The uptake, if not otherwise stated, was terminated by addition of 5 ml of ice-cold 0.15 *M* KCl, followed by immediate filtration through Millipore Hawp filters (diameter = 25 mm; pore size = 0.45 μm). The incubation tubes and filters were further washed twice with ice-cold 0.15 *M* KCl solution. Filters were then dissolved in 10 ml of Filter Count (Packard), and the radioactivity was determined in a Packard Tri-Carb 300 liquid scintillation counter with a counting efficiency of 54–56%. Blanks, treated similarly but incubated at 0°C, were 977 ± 20 (n = 37) and 757 ± 50 cpm (n = 15) (mean ± SEM) for the vesicular and synaptosomal system, respectively. GABA concentration, incubation time, and addition of different metabolic inhibitors had no significant effect on the blank values. The blank values, corresponding to 20–30% of the radioactivity, were retained on the filters under standard GABA uptake conditions.

In each experiment, both samples and blanks were assayed in triplicate, and the mean value was used. In some experiments, the GABA concentration was varied from 44 μ*M* to 10 m*M*, and in others, the incubation time was varied between 90 s and 10 min. When the effect of the metabolic inhibitors CCCP, oligomycin, and ouabain was examined, they were included in the preincubation mixture. CCCP and oligomycin were dissolved in absolute ethanol. The final concentration of ethanol was ~1%, and it had no significant effect on uptake.

Synaptosomal GABA uptake inhibitors (see Table 3) were all added to the preincubation medium. The inhibitor solutions were adjusted to pH 7.4 with NaOH when necessary.

In some experiments, the vesicle fraction was pelleted by centrifugation at 100,000 *g* for 3 h, and the pellet was treated with trichloracetic acid (2.5%) to release the amino acids. The supernatant was extracted with ether to remove trichloracetic acid, then reacted with *o*-phthaldialdehyde under slightly alkaline conditions, and subjected to HPLC as previously described (Lindroth and Mopper, 1979). The amino acid content was determined by fluorescence, and the radioactivity was determined by scintillation counting of 1-ml fractions.

Protein contents in the synaptosome and vesicle preparations were measured as described by Lowry et al. (1951).

Statistics

For uptake studies, the results were expressed as mean ± SEM values. Groups of data were analyzed by Student's *t* test. The K_m and V_{max} values were calculated with a linear regression program (Chou and Chou, 1985).

RESULTS

We have studied the uptake of GABA into a synaptic vesicle fraction. In most experiments, the vesicle fraction (D) was used directly, but in some experiments, a resuspension of the vesicle pellet after centrifugation of the D fraction (diluted with 0.15 *M* KCl) at 100,000 *g* for 3 h was used. The suspension of the vesicle pellet and the D fraction gave similar results, but the D fraction usually gave a higher uptake.

The uptake was stimulated four- or fivefold at 30°C compared with 0°C. The vesicular uptake was, therefore, highly temperature dependent, and uptake at 0°C was taken as the blank throughout the investigation. When the extract from vesicles was reacted with *o*-phthaldialdehyde and subjected to HPLC, the radioactivity traveled with the GABA peak.

When the vesicular fraction was diluted and washed with water instead of 0.15 *M* KCl, uptake was

TABLE 1. *Vesicular uptake of [^{3}H]GABA*

	Treatment	GABA uptake: pmol/min/mg of protein	GABA uptake: %
	Control	9.1 ± 1.2 (8)	100
Minus	ATP	1.6 ± 0.6 (5)[a]	16
Minus	Mg^{2+}	4.8 ± 0.9 (8)[a]	53
Plus	5 μ*M* CCCP	3.6 ± 0.7 (3)[b]	40
Plus	10 μ*M* CCCP	3.4 ± 0.8 (4)[b]	37
Plus	50 m*M* Na^+	8.4 ± 0.8 (6)	92
Plus	2.5 μg of oligomycin	7.6 ± 0.9 (5)	84
Plus	167 μ*M* ouabain	8.3 ± 0.6 (6)	91

A soluble vesicle fraction (D fraction) or a vesicle pellet was incubated in 0.25 *M* sucrose, 5 m*M* Tris-HCl (pH 7.4), 4 m*M* $MgSO_4$, 2 m*M* ATP, and 44 μ*M* [^{3}H]GABA (0.1 Ci/mmol) for 3 min at 30°C. The amount of GABA retained in the vesicles was determined as described in Materials and Methods. Data are mean ± SEM values (no. of determinations).

[a] $p < 0.001$, [b] $p < 0.05$ by Student's *t* test.

reduced by 80%. Under such conditions, the vesicles were osmotically shocked, and accumulated GABA, therefore, leaked out. This confirms that we are dealing with uptake into osmotically sensitive particles rather than with membrane binding.

The uptake was highly dependent on ATP (Table 1). In the absence of ATP, uptake was reduced by 84%. The uptake was also dependent on Mg^{2+}, an observation indicating the involvement of a Mg^{2+}-ATPase. In the presence of small concentrations of the proton carrier CCCP, the ATP-dependent uptake of GABA was inhibited. This indicates the importance of the electrochemical gradient generated by a proton pump ATPase in the synaptic vesicle membranes.

Oligomycin and ouabain had no significant effect on ATP-dependent GABA uptake (Table 1). These agents are known to inhibit the mitochondrial and plasma membrane Na^+,K^+-ATPases, respectively. This confirms that mitochondrial and plasma membrane ATPases were not involved in the GABA uptake described.

The uptake of GABA into the vesicular fraction was compared with that into the synaptosome fraction (Table 2). Unlike vesicular uptake, synaptosomal uptake of GABA was highly stimulated by addition of 50 m*M* NaCl (almost 15-fold). The uptake of GABA into synaptosomes was not reduced by removal of Mg^{2+} or ATP, but it was inhibited by CCCP.

The time course of ATP-dependent GABA uptake up to 10 min is shown in Fig. 1. Maximal uptake was reached after ~5 min of incubation.

The vesicular GABA accumulation in the presence of ATP was saturable with respect to GABA (Fig. 2A). The K_m value for GABA in the presence of ATP was determined to be 5.6 m*M*, and the V_{max} value was 1,500 pmol/min/mg of protein (Fig. 2B).

As shown in Table 3, the uptake of GABA into synaptic vesicles was not inhibited by general, synaptosomal, and glial GABA uptake inhibitors such as nipecotic acid, DABA, or β-alanine. Uptake was not inhibited by L-glutamate or D-aspartate.

TABLE 2. *Accumulation of [^{3}H]GABA by a crude synaptosomal fraction*

	Treatment	GABA uptake pmol/min/mg of protein	%
	Control	98.1 ± 7.5	100
Minus	ATP	99.0 ± 3.9	101
Minus	Mg^{2+}	195.2 ± 27.5[a]	199
Plus	10 μ*M* CCCP	34.0 ± 4.2[b]	34
Minus	50 m*M* Na^+	6.8 ± 2.7[b]	7

A crude synaptosomal pellet (P_2) dissolved in 0.25 *M* sucrose and 5 m*M* Tris-HCl (pH 7.4) was incubated with 4 m*M* $MgSO_4$, 50 m*M* NaCl, 2 m*M* ATP, and 44 μ*M* [^{3}H]GABA (0.1 Ci/mmol) for 3 min at 30°C. The amount of GABA retained in the synaptosomes was determined as described in Materials and Methods. Data are mean ± SEM values from three determinations.

[a] $p < 0.05$, [b] $p < 0.001$ by Student's *t* test.

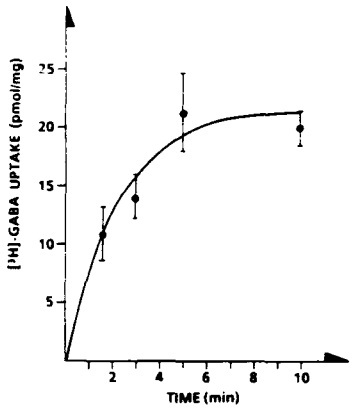

FIG. 1. Time course of [^{3}H]GABA uptake by synaptic vesicles. Synaptic vesicles (D fraction) were incubated in 0.25 *M* sucrose, 5 m*M* Tris-HCl (pH 7.4), 4 m*M* $MgSO_4$, 2 m*M* ATP, and 44 μ*M* [^{3}H]GABA (0.1 Ci/mmol) at 30°C for various times. Each point is the average of three or four separate experiments, and the amount of GABA accumulated in the vesicles was determined as described in Materials and Methods. The bars indicate SEM.

DISCUSSION

In the present study, we provide evidence for a MgATP-dependent uptake system for GABA into synaptic vesicles isolated from rat brain. Accumulation of GABA by synaptic vesicles was highly dependent on temperature. The vesicular system was saturable with respect to time and substrate concentration. Compared with synaptosomal GABA uptake, the affinity and maximal rate were low. Vesicular uptake was inhibited by the proton carrier CCCP, but it was not inhibited by ouabain and oligomycin. Unlike uptake into synaptosomes, vesicular uptake was independent of NaCl and was not inhibited by DABA, β-alanine, or nipecotic acid. GABA accumulated in synaptic vesicles was released under hypoosmotic conditions. Thus, the radioactive GABA retained on the filters was due to uptake rather than binding.

Previously, Naito and Ueda (1983) failed to show any uptake of GABA into immunoprecipitated vesicles. In this preparation, they were only able to show accumulation of glutamate. However, recently, in a preliminary report, they described an ATP-dependent uptake of GABA into synaptic vesicles prepared by Percoll gradient centrifugation (Kish et al., 1987). In the present work, we also provide evidence for a

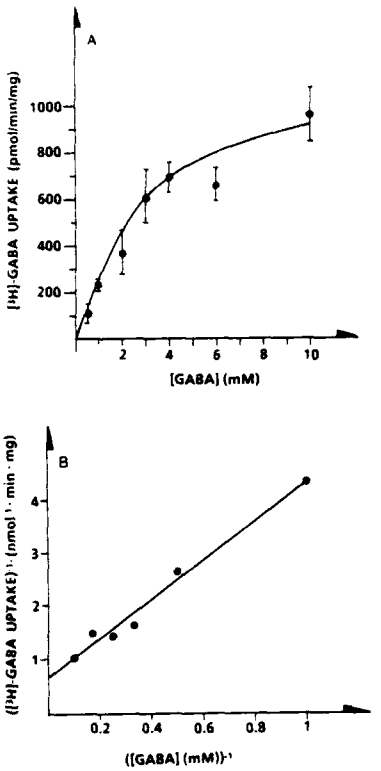

FIG. 2. Substrate dependence of [^{3}H]GABA accumulation by synaptic vesicles. A: Rate of ATP-dependent vesicular uptake of [^{3}H]GABA as a function of GABA concentration. Synaptic vesicles (D fraction) were incubated in 0.25 *M* sucrose, 5 m*M* Tris-HCl (pH 7.4), 4 m*M* $MgSO_4$, and 2 m*M* ATP. The GABA concentration was varied between 500 μM and 10 m*M*, and the amount of GABA retained in the vesicles after 3 min at 30°C was determined as described in Materials and Methods. Each point represents specific GABA uptake (the average of three separate experiments); bars indicate SEM. B: Double reciprocal plot of the data from A. The K_m (5.6 m*M*) and V_{max} (1,500 pmol/min/mg of protein) values were calculated with a linear regression program (Chou and Chou, 1985).

specific vesicular GABA uptake system. There has not yet been any satisfactory evidence for localization of GABA in synaptic vesicles, and the mechanism of storage and release into the synaptic cleft is not clear (De Belleroche and Bradford, 1973; Lahdesmäki et al., 1977). The present results indicate the storage of GABA in synaptic vesicles and, therefore, the possible involvement of synaptic vesicles in GABAergic synaptic transmission. Other criteria need to be fulfilled before this can be firmly established.

Accumulation of GABA by synaptic vesicles isolated from rat brain required ATP hydrolysis and Mg^{2+}. These results are similar to the results of Naito and Ueda (1983, 1985) on glutamate uptake. Their uptake system has been reported to be highly specific for L-glutamate and driven by a vesicle Mg^{2+}-ATPase, generating an electrochemical proton gradient. We have demonstrated that accumulation of GABA by synaptic vesicles from rat brain was significantly decreased in the absence of ATP and Mg^{2+} and in the presence of CCCP, an inhibitor of proton pumps and an uncoupler of oxidative phosphorylation (Heytler and Prichard, 1962). In contrast, oligomycin, a well-known inhibitor of mitochondrial ATPase, did not affect uptake. This indicates that mitochondrial membranes could not be responsible for the vesicular uptake. Ouabain, an agent known to inhibit plasma membrane Na^+,K^+-ATPase and synaptosomal GABA uptake (Nicklas et al., 1973), had no effect on the ATP-dependent vesicular uptake. Because mammalian synaptic vesicles contain an ATP-dependent proton pump (Stadler and Tsukita, 1984), we presume that GABA uptake is driven by a vesicle Mg^{2+}-ATPase, generating an electrochemical proton gradient.

Using synaptic vesicles from the electric organ of *Torpedo*, it has been shown that acetylcholine is also taken up in a saturable MgATP-dependent manner (Koenigsberger and Parsons, 1980; Parsons and Koenigsberger, 1980; Anderson et al., 1982; Parsons et al., 1982). Uncouplers like nigericin, valinomycin, and carbonylcyanid *p*-trifluoromethoxyphenylhydrazone act as potent inhibitors of active acetylcholine uptake. Synaptic vesicles isolated from the rat brain also accumulate [^{3}H]noradrenaline and 5-[^{3}H]-hydroxytryptamine (Seidler et al., 1977; Halaris and DeMet, 1978) in a MgATP-dependent manner.

TABLE 3. *Effects of amino acids and synaptosomal GABA uptake inhibitors on vesicular uptake of [^{3}H]GABA*

Test agent	GABA uptake (pmol/min/mg of protein)
None (control)	11.1 ± 2.0 (5)
β-Ala (10 m*M*)	9.2 ± 0.8 (3)
L-Glu (10 m*M*)	14.3 ± 1.7 (3)
D-Asp (10 m*M*)	13.4 ± 2.0 (3)
DABA (1 m*M*)	13.3 ± 2.2 (3)
Nipecotic acid (1 m*M*)	10.7 ± 1.7 (3)

A soluble vesicle fraction (D fraction) was incubated in 0.25 *M* sucrose, 5 m*M* Tris-HCl (pH 7.4), 4 m*M* $MgSO_4$, 2 m*M* ATP, and 44 μM [^{3}H]GABA (0.1 Ci/mmol). The test agents were included in the preincubation medium. The amount of GABA accumulated by the vesicles was determined as described in Materials and Methods. Data are mean ± SEM values (no. of determinations). The values are not significantly different from the control (Student's *t* test).

These reports agree with our results. Accumulation of neurotransmitters by isolated synaptic vesicles is an active process, probably driven by an electrochemical gradient.

The K_m value determined (5.6 mM) indicates a low-affinity system for GABA uptake into synaptic vesicles. Naito and Ueda (1985) also found a low-affinity K_m value for glutamate uptake (1.6 mM) into synaptic vesicles. The concentration of GABA in the GABAergic terminals has been estimated to be 50–150 mM (Fonnum and Walberg, 1973). A large part of this pool is probably intravesicular, and the concentration in the cytosol may, therefore, be of the same order of magnitude as the K_m of the vesicular uptake. In contrast, Seidler et al. (1977) described a higher affinity for uptake of catecholamine into synaptic vesicles isolated from rat brain.

The described vesicular uptake of GABA differs clearly from that of synaptosomal uptake of GABA with respect to dependence on Na^+ (Martin and Smith, 1972; Kanner, 1978) and ATP (Kanner, 1978).

CCCP, the electrogenic proton carrier, inhibited both the vesicular and the synaptosomal transport systems. The synaptosomal uptake of GABA requires both Na^+ and Cl^- gradients, which are electrogenically maintained (Kanner and Radian, 1986). In the case of synaptosomes, CCCP will, therefore, inhibit the uptake by decreasing the membrane potential (Kanner, 1978).

It is particularly interesting that β-alanine, DABA, and nipecotic acid had no effect on GABA uptake into vesicles. It is well established that DABA and β-alanine are potent inhibitors of synaptosomal and glial uptake, respectively, and that nipecotic acid inhibits both (Iversen and Kelly, 1975; Krogsgaard-Larsen and Johnston, 1975; Schon and Kelly, 1975).

In conclusion, the vesicular uptake of GABA is driven by a Mg^{2+}-ATPase coupled to an electrogenic pump. The vesicular uptake system is clearly different from those of glia and synaptosomes.

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PAPER II

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Comparison of the Properties of γ-Aminobutyric Acid and L-Glutamate Uptake into Synaptic Vesicles Isolated from Rat Brain

Else M. Fykse, Hege Christensen, and Frode Fonnum

Division for Environmental Toxicology, Norwegian Defence Research Establishment, Kjeller, Norway

Abstract: Rat brain synaptic vesicles exhibit ATP-dependent uptake of γ-[^{3}H]amino-*n*-butyric acid ([^{3}H]GABA) and L-[^{3}H]glutamate. After hypotonic shock, the highest specific activities of uptake of both L-glutamate and GABA were recovered in the 0.4 *M* fraction of a sucrose gradient. The uptakes of L-glutamate and GABA were inhibited by similar, but not identical, concentrations of the mitochondrial uncoupler carbonyl cyanide *m*-chlorophenylhydrazone and the ionophores nigericin and gramicidin, but they were not inhibited by the K^+ carrier valinomycin. *N,N'*-Dicyclohexylcarbodiimide and *N*-ethylmaleimide, Mg^{2+}-ATPase inhibitors, inhibited the GABA and L-glutamate uptakes similarly. Low concentrations of Cl^- stimulated the vesicular uptake of L-glutamate but not that of GABA. The uptakes of both L-glutamate and GABA were inhibited by high concentrations of Cl^-. These results indicate that the vesicular GABA and L-glutamate uptakes are driven by an electrochemical proton gradient generated by a similar Mg^{2+}-ATPase. The vesicular uptake mechanisms are discussed in relation to other vesicle uptake systems. **Key Words:** Synaptic vesicles—Vesicular uptake—Mg^{2+}-ATPase—Proton gradient—Inhibitors. **Fykse E. M. et al.** Comparison of the properties of γ-aminobutyric acid and L-glutamate uptake into synaptic vesicles isolated from rat brain. *J. Neurochem.* **52**, 946–951 (1989).

γ-Amino-*n*-butyric acid (GABA) and L-glutamate are important neurotransmitters in the CNS (Krnjevic, 1970; Fonnum, 1987). However, the mechanisms by which the amino acid neurotransmitters are stored and released within the nerve terminal are still elusive. Until now, no one has been able to show any enrichment of endogenous GABA and L-glutamate in isolated synaptosomes or synaptic vesicle preparations (De Belleroche and Bradford, 1973; Lahdesmaki et al., 1977; Wood and Kurylo, 1984). Active uptake of both L-glutamate and GABA has, however, been demonstrated with different preparations of mammalian synaptic vesicles (Philippu and Matthaei, 1975; Disbrow et al., 1982; Naito and Ueda, 1983; Fykse and Fonnum, 1988). The vesicular uptake of GABA and L-glutamate did not require Na^+ and was highly dependent on Mg^{2+} and ATP. The uptake was not inhibited by inhibitors of glial and synaptosomal uptake. The vesicular uptake was, therefore, clearly different from that of glial cells and synaptosomes (Naito and Ueda, 1985; Fykse and Fonnum, 1988).

Different vesicle preparations show different affinities toward L-glutamate uptake (Disbrow et al., 1982; Naito and Ueda, 1983, 1985) and very different activity toward GABA (Naito and Ueda, 1983; Kish et al., 1987). Preliminary results have shown that the ratio between L-glutamate and GABA uptake differs for different brain regions, an observation indicating that GABA and L-glutamate are taken up by different vesicles (Fonnum et al., 1988). Therefore, it was of interest to compare the uptake of GABA and L-glutamate in a similar vesicle preparation.

In this article, we have compared in detail the effect of one proton gradient uncoupler, ionophores, and Mg^{2+}-ATPase inhibitors on a vesicle preparation that contains both GABAergic and glutamergic vesicles and that shows a high ratio between GABA and L-glutamate uptake. In fact, we present the highest GABA/L-glu-

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Address correspondence and reprint requests to Dr. E. M. Fykse at Division for Environmental Toxicology, Norwegian Defence Research Establishment, P.O. Box 25, N-2007 Kjeller, Norway.

Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; GABA, γ-amino-*n*-butyric acid; NEM, *N*-ethylmaleimide.

tamate uptake ratio (1:4) shown. The results are discussed in relation to other vesicular uptake systems.

MATERIALS AND METHODS

GABA, L-glutamate (dipotassium salt), ATP (disodium salt), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), *N,N'*-dicyclohexylcarbodiimide (DCCD), nigericin, and valinomycin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [2,3-^{3}H]GABA (45 Ci/mmol) and L-[2,3-^{3}H]glutamate (25 Ci/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.). *N*-Ethylmaleimide (NEM) was from Nutritional Biochemicals Corp. (Cleveland, OH, U.S.A.).

Purification of synaptic vesicles

Male Wistar rats (Møllegaard, Denmark), weighing 200–250 g, were used in all experiments. For each experiment, brains from ~10 rats were removed after decapitation. The subcellular fractionation was carried out as described by Whittaker et al. (1964), except that 10 m*M* Tris-maleate (pH 7.4) and 1.0 m*M* EGTA were included in the sucrose solution (Stadler and Tsukita, 1984). The crude synaptosomal fraction (P_2) was osmotically shocked by resuspension in 10 m*M* Tris-maleate (pH 7.4) and 0.1 m*M* EGTA and centrifuged at 17,000 *g* for 30 min. The remaining supernatant was subjected to sucrose density gradient centrifugation in a Contron TST.28.38 rotor at 65,000 *g* for 2 h, and the vesicle fraction was isolated from the band containing 0.4 *M* sucrose.

Assay for GABA and L-glutamate uptake

Vesicular GABA and L-glutamate uptakes were determined as described by Fykse and Fonnum (1988), except that the standard incubation mixture for assaying vesicular uptake contained 110 m*M* potassium tartrate, 10 m*M* Tris-maleate (pH 7.4), and 4 m*M* $MgCl_2$. Synaptic vesicles (~0.1 mg of protein) were incubated with 1 m*M* [^{3}H]GABA or L-[^{3}H]glutamate (5 mCi/mmol) and 2 m*M* ATP (disodium salt neutralized with Tris base). The vesicles were incubated at 30°C for 3 min. The reaction was stopped by filtration through a Millipore HAWP filter (diameter = 24 mm; pore size = 0.45 μm), and the radioactivity was determined in a Packard Tri-Carb 2200 Liquid Scintillation counter with a counting efficiency of 56–58%. Blanks were incubated at 0°C and were 328 ± 13 cpm (n = 81) and 475 ± 18 cpm (n = 82) for the vesicular GABA and L-glutamate uptake systems, respectively. Addition of different metabolic inhibitors had no significant effect on the blank values, which corresponded to ~15–20 and 5–10% of the radioactivity that was retained on the filters under standard GABA and L-glutamate uptake conditions, respectively.

When the effect of the metabolic inhibitors CCCP, DCCD, valinomycin, nigericin, gramicidin, and NEM were examined, they were included in the preincubation mixture. The inhibitors were dissolved in absolute ethanol. The final concentration of ethanol was 1%. Control experiments showed that this concentration had no significant effect on the uptake.

When the effect of Cl^- was studied, the vesicle fraction was eluted through a Sephadex G-25 column (Pharmacia PD-10) before incubation to reduce the Cl^- concentration in the vesicle fraction.

Protein contents were measured as described by Lowry et al. (1951).

The results were expressed as mean ± SEM values. Groups of data were analyzed by Student's *t* test. The IC_{50} values were calculated from three different experiments with a Multiple Drug Effect Analysis Program (Chou and Chou, 1985).

RESULTS

Fractionation of synaptic vesicles on a discontinuous sucrose gradient

We have studied uptake of GABA and L-glutamate into synaptic vesicles fractionated on a discontinuous sucrose gradient. Specific activity of the ATP-dependent GABA and L-glutamate uptake was highest in the 0.4 and 0.6 *M* sucrose bands (Fig. 1). Uptakes of GABA

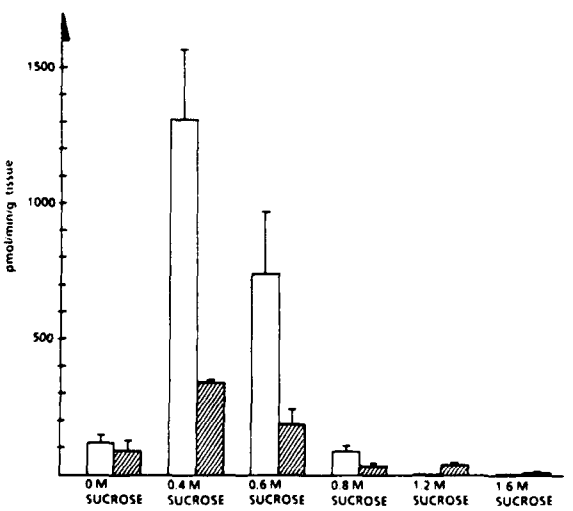

FIG. 1. Vesicular uptake of GABA (▨) and L-glutamate (□) in a sucrose gradient. The incubation mixture contained 0.32 *M* sucrose, 10 m*M* Tris-maleate (pH 7.4), 4 m*M* $MgCl_2$, 2 m*M* ATP, and 1 m*M* L-[^{3}H]glutamate or [^{3}H]GABA (5 mCi/mmol). Data are mean ±SD (bars) values from two different experiments.

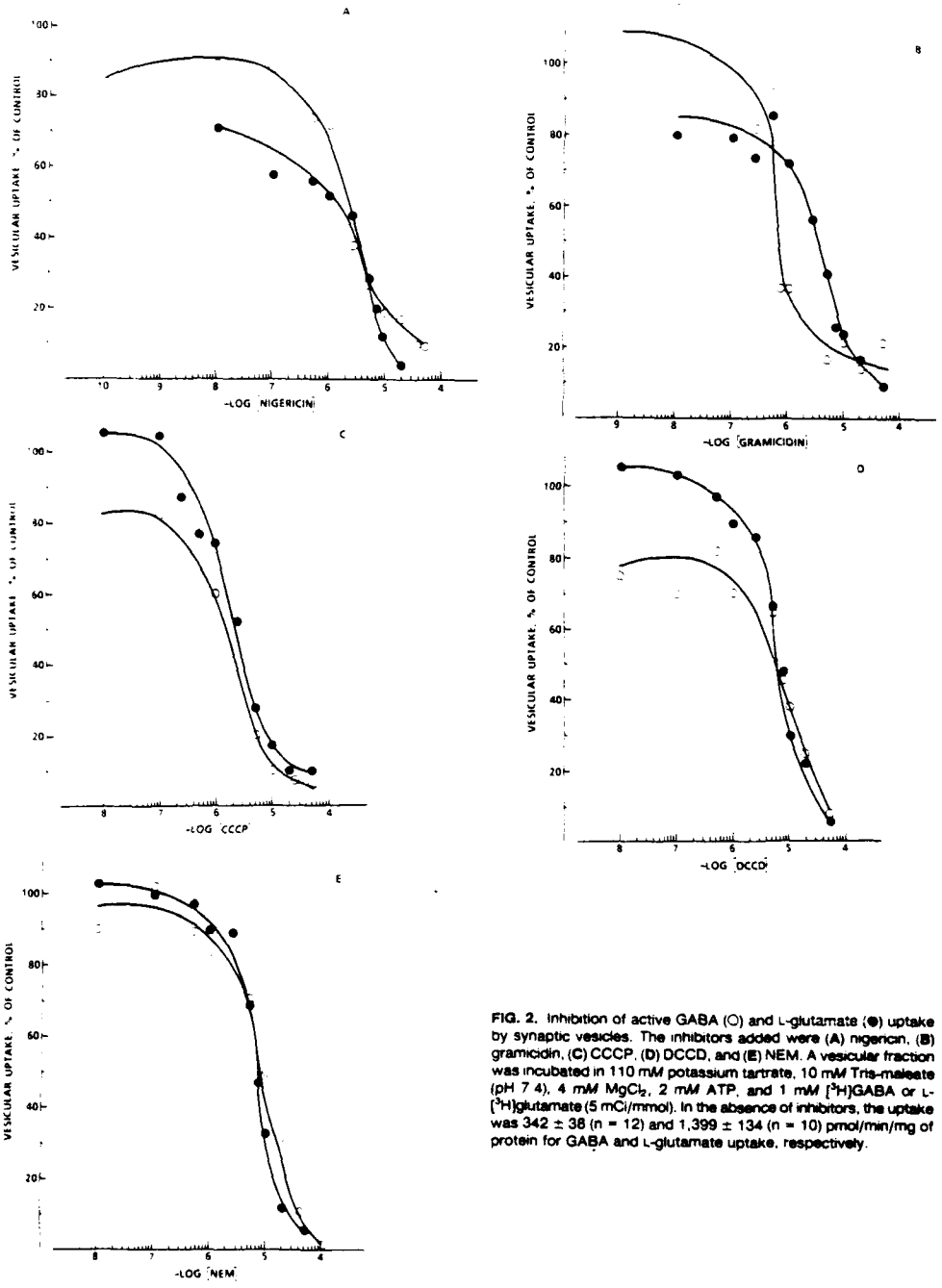

FIG. 2. Inhibition of active GABA (○) and L-glutamate (●) uptake by synaptic vesicles. The inhibitors added were (A) nigericin, (B) gramicidin, (C) CCCP, (D) DCCD, and (E) NEM. A vesicular fraction was incubated in 110 m*M* potassium tartrate, 10 m*M* Tris-maleate (pH 7.4), 4 m*M* $MgCl_2$, 2 m*M* ATP, and 1 m*M* [^{3}H]GABA or L-[^{3}H]glutamate (5 mCi/mmol). In the absence of inhibitors, the uptake was 342 ± 38 (n = 12) and 1,399 ± 134 (n = 10) pmol/min/mg of protein for GABA and L-glutamate uptake, respectively.

and L-glutamate in the high-speed supernatant loaded on the gradient (see Materials and Methods) were 109 ± 59 (n = 2) and 314 ± 90 (n = 2) pmol/min/mg of protein (mean ± SD), and in the 0.4 *M* sucrose band the GABA and L-glutamate uptakes were 446 ± 111 (n = 2) and 1,670 ± 63 (n = 2) pmol/min/mg of protein (mean ± SD), respectively. Specific GABA and L-glutamate uptakes were about four and five times higher in the 0.4 *M* sucrose fraction. The uptake was different from the uptake into membranes, and the H fraction, containing a mixture of membrane-fused vesicles and disrupted synaptosomes, did not show any uptake.

Effects of different inhibitors on ATP-dependent GABA and L-glutamate uptake

Purified rat brain synaptic vesicles were incubated with a wide concentration range of different classes of mitochondrial uncouplers to examine their effect on the ATP-dependent GABA and L-glutamate uptake.

Figure 2 shows the effect of nigericin, gramicidin, CCCP, DCCD, and NEM on the uptake of GABA and L-glutamate. The IC_{50} values are given in Table 1. The electroneutral H^+–K^+ or H^+–Na^+ exchanger nigericin, in the presence of 110 m*M* KCl and 4 m*M* NaCl, acted as an inhibitor of active uptake of GABA and L-glutamate. The half-maximal inhibitory concentrations were 2×10^{-6} and 0.3×10^{-6} *M*, respectively. Thus, the inhibition of the L-glutamate uptake was more potent. In the absence of K^+, the IC_{50} values were >50 μM (data not shown).

The channel-former gramicidin, which would allow free movement of H^+, K^+, and Na^+, also inhibited active uptake of GABA and L-glutamate. The IC_{50} values for the GABA and L-glutamate uptake were 0.8×10^{-6} and 3.2×10^{-6} *M*, respectively. In this case, the inhibition of the GABA uptake was more potent than that of L-glutamate uptake.

The electrogenic proton carrier CCCP completely inhibited active uptake of GABA and L-glutamate with almost identical IC_{50} values (Table 1).

TABLE 1. *Effect of different inhibitors on uptake of GABA and L-glutamate*

Inhibitor	IC_{50} (μM)	
	GABA	L-Glutamate
Nigericin	2.0	0.3
Gramicidin	0.8	3.2
CCCP	1.6	2.2
DCCD	5.4	6.4
NEM	7.2	7.2
Valinomycin	>50	>50

A vesicle fraction was incubated in 110 m*M* potassium tartrate, 10 m*M* Tris-maleate (pH 7.4), 4 m*M* $MgCl_2$, 2 m*M* ATP, and 1 m*M* [^{3}H]GABA or L-[^{3}H]glutamate (5 mCi/mmol). The IC_{50} values were calculated with a Multiple Drug Effect Analysis Program (Chou and Chou, 1985).

TABLE 2. *Effect of KCl on vesicular uptake of GABA and L-glutamate*

KCl (m*M*)	Vesicular uptake [Relative activity (%)]	
	GABA	L-Glutamate
5	99 ± 12 (4)	569 ± 110 (6)[a]
50	87 ± 23 (4)	165 ± 43 (6)
100	65 ± 14 (4)[b]	76 ± 23 (6)

A vesicle fraction eluted through a Sephadex G-25 (PD-10) column was incubated in 110 m*M* potassium tartrate, 10 m*M* Tris-maleate (pH 7.4), 4 m*M* $MgSO_4$, 2 m*M* ATP, and 1 m*M* L-[^{3}H]glutamate or [^{3}H]GABA (5 mCi/mmol). The control values in the absence of KCl were 372 ± 38 (n = 7) and 268 ± 63 (n = 7) pmol/min/mg of protein for the GABA and L-glutamate uptake, respectively. Data are mean ± SEM percentages relative to the control (no. of determinations).

The significance of differences was calculated by Student's *t* test: [a]$p < 0.002$, [b]$p < 0.05$.

For further examination of the importance of the Mg^{2+}-ATPase and the proton gradient, ATP-dependent uptakes of GABA and L-glutamate were studied with different concentrations of the Mg^{2+}-ATPase inhibitor DCCD. The inhibition was nearly identical.

NEM, a thiol reagent and a proton pump ATPase inhibitor, caused a potent inhibition of the GABA and L-glutamate uptake. This means that one or more reduced cysteine residue(s) are important in the ATP-dependent vesicular GABA and L-glutamate uptake.

The K^+ carrier valinomycin showed nearly no inhibition of the GABA and L-glutamate uptake at 110 m*M* K^+. The uptakes of L-glutamate and GABA were 77 ± 7% (n = 4) and 71 ± 11% (n = 4), respectively, of the control value in the presence of 50 μM valinomycin.

Effect of chloride

The effect of different Cl^- concentrations on the ATP-dependent vesicular uptake was examined (Table 2). Synaptic vesicles were eluted through a Sephadex G-25 (PD-10) column to reduce the Cl^- concentration. Uptake of GABA and L-glutamate with different concentrations of Cl^- added was examined in the eluate. The results are shown in Table 2. Addition of 5 m*M* Cl^- had no effect on the GABA uptake, whereas the L-glutamate uptake was stimulated ~500%. High concentrations of Cl^- inhibited both the GABA and L-glutamate uptake. In the absence of Cl^-, the uptake of GABA was 140% compared with the L-glutamate uptake. When 5 m*M* Cl^- was added, the GABA uptake was ~25% compared with the L-glutamate uptake. To determine whether this difference was an effect of Cl^-, we examined the effect of NaCl and K_2SO_4 (data not shown). NaCl at 5 m*M* stimulated the uptake of L-glutamate, whereas 5 m*M* K_2SO_4 did not have any effect. Addition of 5 m*M* NaCl and K_2SO_4 did not affect the uptake of GABA.

DISCUSSION

In the present study, we have examined mechanisms of the Mg^{2+}-ATP-dependent uptake for GABA and L-glutamate into synaptic vesicles, isolated from rat brain by sucrose gradient centrifugation. The uptake of GABA and L-glutamate was concentrated in the 0.4 *M* sucrose layer. Vesicular uptake was inhibited by the K^+–H^+ or the Na^+–H^+ exchanger nigericin and the channel-former gramicidin, which would allow movement of H^+, K^+, or Na^+. The proton carrier CCCP and the Mg^{2+}-ATPase inhibitor DCCD inhibited the uptake, but the vesicular uptake was not inhibited by the K^+ carrier valinomycin. The vesicular uptake was inhibited by the SH-group blocking agent NEM. Addition of low concentrations of KCl stimulated the uptake of L-glutamate, whereas the uptake of GABA was not affected. The ratio between GABA and L-glutamate uptake (1:4) was the highest ever reported.

The experiments presented in this article support the proposed involvement of a membrane-bound Mg^{2+}-ATPase and a transmembrane pH gradient in the uptake of L-glutamate and GABA into synaptic vesicles. These results confirm and extend previous reports that vesicular uptakes of GABA and L-glutamate are driven by a proton gradient generated by a Mg^{2+}-ATPase (Naito and Ueda, 1983, 1985; Kish et al., 1987; Fykse and Fonnum, 1988). Stadler and Tsukita (1984) examined the properties of an ATPase in brain vesicles and even investigated it ultrastructurally. Uptake of acetylcholine into synaptic vesicles from the electric organ of *Torpedo* (Anderson et al., 1982) and uptakes of noradrenaline and 5-hydroxytryptamine into synaptic vesicles isolated from rat brain (Seidler et al., 1977; Halaris and DeMet, 1978) are dependent on Mg^{2+}, ATP, and also a proton gradient. Thus, accumulation of neurotransmitters by synaptic vesicles is an active process driven by a proton gradient.

Naito and Ueda (1985) examined the effect of nigericin, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (an uncoupler similar to CCCP), and NEM on L-glutamate uptake into immunoprecipitated vesicles from bovine brain. Their results are in agreement with ours. Thus, synaptic vesicles isolated from different animals by different preparation techniques behaved in a similar manner with respect to these inhibitors.

DCCD inhibits the mitochondrial ATPase (Beechey et al., 1966) and the Mg^{2+}-ATPase activity of synaptic vesicles (Toll et al., 1977; Toll and Howard, 1978). Toll and Howard (1978) also found that DCCD caused a potent inhibition of the noradrenaline uptake. In addition, DCCD inhibits the uptake of GABA and L-glutamate, and the IC_{50} values are nearly the same. The thiol reagent NEM, which inhibits the proton pump activity of chromaffin granules (Flatmark et al., 1982, 1985), inhibited the GABA and L-glutamate uptake to the same extent.

Uncouplers of oxidative phosphorylation render membranes permeable to protons. The uncoupler CCCP causes an equilibration of protons across the vesicle membrane, thus destroying the electrochemical potential (Heytler and Prichard, 1962). CCCP inhibited the GABA and L-glutamate uptake.

Nigericin caused a potent inhibition of both the GABA and L-glutamate uptake. Nigericin produces an electroneutral exchange of K^+ and H^+ in the presence of K^+. Protons and K^+ will travel down their concentration gradients (Toll and Howard, 1978).

As expected, gramicidin also rendered the synaptic vesicles unable to accept GABA and L-glutamate. Gramicidin forms a channel across the membrane, and ions like H^+, K^+, and Na^+ are able to pass. In contrast to nigericin, gramicidin caused a more potent inhibition of the GABA uptake than the L-glutamate uptake. This may reflect the different charge of GABA and L-glutamate; thus, different ions will affect the GABA and L-glutamate uptake differently. Toll and Howard (1978) found that nigericin and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone caused a potent inhibition of the vesicular uptake. These results strongly support the hypothesis that a transmembrane pH gradient, generated by a Mg^{2+}-ATPase, is utilized in the uptake of GABA and L-glutamate into synaptic vesicles. In addition, uptake of GABA is insensitive to oligomycin and ouabain (Fykse and Fonnum, 1988), agents known to inhibit the mitochondrial H^+- and plasma membrane Na^+,K^+-ATPase, respectively. Thus, the vesicular Mg^{2+}-ATPase is different from these enzymes. The vesicular Mg^{2+}-ATPase belongs to a class of ATP-driven ion pumps very similar to that described in endosomes, lysosomes, coated vesicles, and plant vacuoles (Rudnick, 1986; Kanner and Schuldiner, 1987).

The K^+ carrier valinomycin did not inhibit the GABA and L-glutamate uptake. Valinomycin is an electrogenic K^+ carrier that should alter an electrical potential across synaptic vesicle membranes without affecting the pH gradient (Johnson and Scarpa, 1976). Toll and Howard (1978) did not observe any effect of valinomycin on the vesicular noradrenaline uptake. In contrast, the results of Anderson et al. (1982) showed that valinomycin caused a potent inhibition of acetylcholine accumulation by synaptic vesicles isolated from *Torpedo* electric organ. Their results indicate that a part of the energy-driving acetylcholine uptake probably is electrical in nature.

Addition of 5 m*M* Cl^- caused a large increase of the L-glutamate uptake. In contrast, the uptake of GABA was not increased by addition of 5 m*M* Cl^-. This is in agreement with the results of Naito and Ueda (1985) on L-glutamate. Moriyama and Nelson (1987) have purified an ATPase from chromaffin granule membranes. They showed that this enzyme is an anion-dependent proton pump. The ATP-dependent proton uptake activity of the reconstituted enzyme was ab-

solutely dependent on the presence of Cl^- or Br^- in low concentrations. Because it is not possible to reduce the Cl^- concentration to 0, these results do not exclude an effect of extremely low concentration of Cl^- on the uptake of GABA. More experiments have to be done before this can be firmly stated.

In conclusion, the vesicular uptakes of both GABA and L-glutamate have been shown to be driven by Mg^{2+}-ATPase proton pumps. The observed difference between the GABA and L-glutamate uptake may reflect the different charge of GABA and L-glutamate or different properties of their Mg^{2+}-ATPase proton pumps.

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PAPER III

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Transport of γ-aminobutyrate and L-glutamate into synaptic vesicles

Effect of different inhibitors on the vesicular uptake of neurotransmitters and on the Mg^{2+}-ATPase

Else M. FYKSE* and Frode FONNUM
Norwegian Defence Research Establishment, Division for Environmental Toxicology, P.O. Box 25, N-2007 Kjeller, Norway

The uptakes of γ-aminobutyrate (GABA) and L-glutamate into synaptic vesicles isolated from rat brain were compared with respect to the effects of 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonic acid (SITS), 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid (DIDS) and 5-nitro-2-(3-phenylpropylamino)benzoic acid (N144), agents known to block anion channels. The uptake of glutamate was inhibited by low micromolar concentrations of SITS, DIDS and N144. GABA uptake was much less sensitive to these agents than was glutamate uptake. SITS and N144 inhibited the vacuolar H^+-ATPase of synaptic vesicles to a smaller extent than the glutamate uptake. The uptake of GABA was not affected by the permeant anions Cl^- and Br^-, whereas the uptake of glutamate was highly stimulated by low concentrations of these ions. The uptakes of both glutamate and GABA were inhibited by similar, but not identical, concentrations of the lipophilic anion SCN^-.

INTRODUCTION

Mammalian brain synaptic vesicles actively accumulate γ-aminobutyrate (GABA), glycine and glutamate by a Mg^{2+}–ATP-dependent process. A vesicular Mg^{2+}-ATPase generates an electrochemical proton gradient which is important for uptake of neurotransmitters (Disbrow *et al.*, 1982; Naito & Ueda, 1985; Fykse & Fonnum, 1988; Hell *et al.*, 1988; Maycox *et al.*, 1988; Kish *et al.*, 1989; Christensen *et al.*, 1990). The Mg^{2+}-ATPase of synaptic vesicles belongs to a class of vacuolar H^+-ATPases which are responsible for acidification of different, subcellular organelles (Nelson, 1986, 1987; Maycox *et al.*, 1988; Cidon & Sihra, 1989; Shioi *et al.*, 1989; Floor *et al.*, 1990). GABA and glutamate are taken up into different populations of synaptic vesicles (Fykse & Fonnum, 1989) with slightly different affinities. The K_m value for the uptake of glutamate was about 1 mM (Naito & Ueda, 1985; Maycox *et al.*, 1988), and the K_m value for GABA was determined to about be about 6 mM (Fykse & Fonnum, 1988; Kish *et al.*, 1989). The evidence for a specific vesicular uptake of GABA, glycine and glutamate supports the notion that synaptic vesicles play an important role in amino acid synaptic transmission.

The uptake of glutamate is stimulated by low concentrations of Cl^- (Naito & Ueda, 1985; Maycox *et al.*, 1988; Fykse *et al.*, 1989), whereas the uptake of GABA is insensitive to variations in the concentration of Cl^- (Fykse *et al.*, 1989; Kish *et al.*, 1989). Different groups have reported that ATP hydrolysis generated a large proton gradient across the synaptic-vesicle membrane at high concentrations of Cl^-. A small proton gradient and a large membrane potential are reported at low Cl^- concentrations (Maycox *et al.*, 1988; Cidon & Sihra, 1989). The uptake of glutamate is driven by the membrane potential, since collapsing the membrane potential by high concentrations of Cl^- inhibited the uptake of glutamate (Maycox *et al.*, 1988; Cidon & Sihra, 1989; Shioi *et al.*, 1989). On the basis of these experiments, a direct involvement of Cl^- in the uptake of glutamate would be a reasonable explanation. The purpose of the present study was to investigate the differences in the mechanisms of the uptake of GABA and glutamate in detail, and specially to examine the role of Cl^- and other permeant ions in the uptake of GABA and glutamate. We have compared the effect of the anion-channel blockers 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid (DIDS), 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonic acid (SITS) and 5-nitro-2-(3-phenylpropylamino)benzoic acid (N144) on the uptake of GABA and glutamate and on the vesicular Mg^{2+}-ATPase activity.

EXPERIMENTAL

Materials

GABA, L-glutamate (dipotassium salt), ATP (disodium salt), SITS, DIDS and CPG-3000 controlled-pore glass beads of nominal pore diameter 271.7 nm (lot 36F-0650, mesh 120/200) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [2,3-^{3}H]GABA (40 Ci/mmol) and L-[2,3-^{3}H]glutamate (25 Ci/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.). N144 was a gift from Dr. J. J. Nordmann, Centre de Neurochimie, Strasbourg, France.

Preparation of synaptic vesicles

Synaptic vesicles were purified from male Wistar rats (200–250 g) obtained from Møllegaard, Ejby, Denmark. For each experiment, 10–15 rats were killed by decapitation, and the brains were quickly removed and kept on ice. Synaptic vesicles were isolated as described in principle by Whittaker *et al.* (1964) and in detail by Fykse & Fonnum (1988). The crude synaptosomal fraction (P_2) was osmotically shocked by resuspension in 10 mM-Tris/maleate (pH 7.4)/0.1 mM-EGTA and centrifuged at 13000 *g* for 30 min. The supernatant was laid on the top of 0.4 M- and 0.6 M-sucrose solutions and centrifuged in a Contron TST 28.38 rotor at 65000 *g* for 2 h. The vesicle fraction was isolated from the band containing 0.4 M-sucrose (D-fraction). The vesicle preparations were stored in liquid N_2 without loss of activity. This vesicle preparation was used in most of the uptake experi-

Abbreviations used: AChE, acetylcholinesterase (EC 3.1.1.7); DIDS, 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid; GABA, γ-amino-n-butyric acid; IC_{50}, concn. giving 50% inhibition; NEM, *N*-ethylmaleimide; N144, 5-nitro-2-(3-phenylpropylamino)benzoic acid; SITS, 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonic acid.

* To whom correspondence should be addressed.

ments. The vesicular uptake was not stimulated by Na^+ (Fykse & Fonnum, 1988). Any contamination by plasma membranes therefore cannot be of any significance. Furthermore, the uptake was dependent on Mg^{2+} and ATP and inhibited by proton ionophores (Fykse *et al.*, 1989).

To compare the effect of the inhibitors on the Mg^{2+}-ATPase activity and the uptake of GABA and glutamate, the D-fraction was further purified by chromatography on a column (44 cm × 1.6 cm) of CPG-3000 controlled-pore glass beads in 110 mM-potassium tartrate/10 mM-Hepes (pH 7.4)/0.1 mM-EGTA. The elution rate was 1 ml/min, and 3 ml fractions were collected.

Vesicular uptake of GABA and glutamate

This was done mainly as described by Fykse & Fonnum (1988). The standard incubation mixture (total volume 0.3 ml) for assaying vesicular uptake contained 0.25 M-sucrose, 10 mM-Tris/maleate (pH 7.4) and 4 mM-$MgCl_2$ (if not otherwise stated). Synaptic vesicles (about 0.1 mg of protein) were preincubated at 30 °C for 15 min, and [^{3}H]GABA (1 μCi; final concn. 1 mM) or L-[^{3}H]glutamate (0.5 μCi; final concn. 1 mM) and ATP (final concn. 2 mM; disodium salt neutralized by Tris base) were added, and the mixture was further incubated for 3 min at 30 °C. The reaction was stopped by addition of 7 ml of ice-cold 0.15 M-KCl, followed by rapid filtration through a Millipore HAWP filter (diameter 25 mm, pore size 0.45 μm). The incubation tubes were further washed twice with the KCl solution. The filters were dissolved in 10 ml of Filter Count (Packard), and the radioactivity was determined in a Packard Tri-Carb 2200 liquid-scintillation counter with a counting efficiency of 50–56 %. Blanks were identical with the test solution in each case, but were incubated at 0 °C. Blank values corresponded to about 10 and 15 % of the radioactivity retained on the filters for the uptakes of glutamate and GABA, and the blank values were 266 ± 10 and 434 ± 19 c.p.m. (both $n = 20$) respectively. The different inhibitors and anions tested were included in the preincubation mixtures.

Uptakes of GABA and glutamate were also studied on the fractions eluted from the CPG-3000 controlled-pore glass column. In these experiments the uptakes of GABA and glutamate were measured in the elution buffer, which also was used for the Mg^{2+}-ATPase experiments.

Assays for Mg^{2+}-ATPase activity, acetylcholinesterase (AChE) activity and protein

The Mg^{2+}-ATPase activity was measured at 30 °C mainly as described by Penefsky & Bruist (1984). The incubation mixture for measuring Mg^{2+}-ATPase contained synaptic vesicles (10–40 μg of protein), 10 mM-Hepes (pH 7.4), 110 mM-potassium tartrate, 4 mM-$MgCl_2$, 2 mM-phosphoenolpyruvate, 20 units each of lactate dehydrogenase and pyruvate kinase/ml, 0.06 mM-K_2NADH and 3 mM-ATP (pH 7.4) in a total volume of 1225 μl. The conversion of NADH into NAD^+ was measured at 340 nm in a Beckman DU 50 spectrophotometer, and 1 nmol of NADH converted corresponds to 1 nmol of phosphate used.

The AChE activity was measured by a radiochemical method (Sterri & Fonnum, 1978). The protein content were measured as described by Lowry *et al.* (1951), with BSA as standard.

Statistics

The results are expressed as mean values (± S.E.M.) of absolute uptake or as relative uptakes (as percentage of controls). Groups of data were analysed by Student's *t* test. The IC_{50} values (concn. giving 50 % inhibition) were calculated from 3 or 4 different experiments with a Multiple Drug Effect Analysis Program (Chou & Chou, 1985).

RESULTS

Effect of inhibitors on vesicular uptake

Owing to the ability of anions to influence vesicular glutamate uptake activity, the effects of SITS and DIDS, agents known to affect plasma-membrane anion channels (Cabantchik *et al.*, 1978), were examined (Figs. 1*a* and 1*b*). Both compounds inhibited the uptake of glutamate better than the uptake of GABA. The IC_{50} values for the uptake of glutamate were calculated as 1.4 μM for DIDS and 3.1 μM for SITS. For the uptake of GABA, the IC_{50} values for DIDS and SITS were calculated as 9.9 μM and 40 μM respectively. In the absence of Cl^- the effect of SITS on the uptake of glutamate was decreased, and the IC_{50} value was calculated as 13 μM. The effect of SITS on the uptake of GABA was not affected by removing Cl^-. In crude synaptosomal fractions of rat brains, the IC_{50} values of SITS for the high-affinity uptake of GABA and glutamate were about 1 mM (E. M. Fykse, unpublished work). This is consistent with results of Fosse *et al.* (1989) on glutamate uptake. Similar results were obtained with the more specific Cl^--channel blocker N144 (Wangemann *et al.*, 1986) on the vesicular uptake of GABA and glutamate (Fig. 1*c*). The IC_{50} value for glutamate uptake was calculated as 15 μM, whereas for GABA it was calculated as 150 μM.

Thiocyanate anion (SCN^-) is membrane-permeant and is

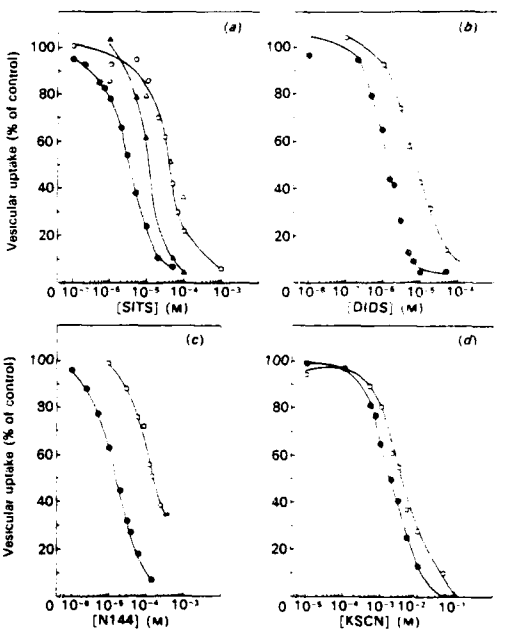

Fig. 1. Effects of SITS, DIDS, N144 and KSCN on the uptake of GABA and glutamate

The activity was assayed as stated in the Experimental section. SITS (*a*), DIDS (*b*), N144 (*c*) and KSCN (*d*) were added just before the preincubation, which lasted for 15 min. In the absence of inhibitors, the uptakes of GABA and glutamate in the presence of 8 mM-Cl^- were 820 ± 80 ($n = 13$) and 2900 ± 200 ($n = 19$) pmol/min per mg of protein respectively; in the absence of Cl^- the glutamate control value was 500 ± 100 pmol/min per mg of protein ($n = 5$) (n = no. of experiments). Key: ●, glutamate (+Cl^-); ▲, glutamate (−Cl^-); ○, GABA (+Cl^-); △, GABA (−Cl^-).

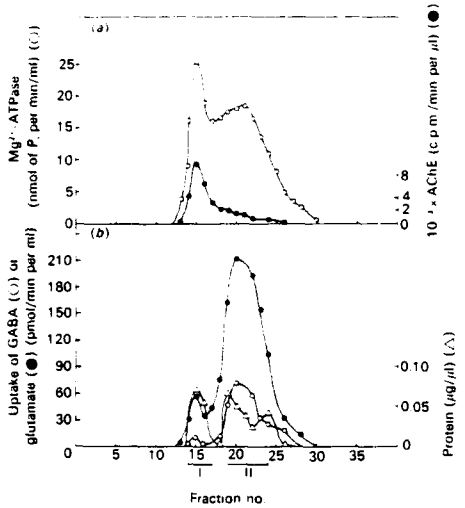

Fig. 2. Chromatography of synaptic vesicles on a column of CPG-3000 controlled-pore glass beads

The recovery of protein was 51%. (*a*) Distribution of Mg^{2+}-ATPase and AChE. (*b*) Distribution of the uptakes of GABA and glutamate and protein content. I and II refer to the pooled fractions mentioned in the Results section.

Table 1. Effects of inhibitors on the Mg^{2+}-ATPase activity

The D-fraction was purified by chromatography on a controlled-pore glass column (CPG-3000). Fractions 14–17 (I) and 19–24 (II) were pooled, and the Mg^{2+}-ATPase activity was measured as described in the Experimental section. In all experiments, 50 μM-vanadate, 1 mM-ouabain and 5 μg of oligomycin B/ml were included. The enzyme activity is expressed as % of control, and the data are means ± S.E.M. of three independent experiments. The control values were 190 ± 50 and 180 ± 20 nmol of P_i/min per mg of protein for fractions I and II respectively. The significance of difference was calculated by Student's *t* test: $^{*}P < 0.05$, $^{**}P < 0.005$.

	Mg^{2+}-ATPase [relative activity (%)]	
Inhibitor	Fraction I (14–17)	Fraction II (19–24)
SITS (1 μM)	101 ± 8	95 ± 5
SITS (10 μM)	90 ± 8	83 ± 1*
SITS (25 μM)	80 ± 11	69 ± 6*
N144 (1 μM)	102 ± 7	103 ± 3
N144 (10 μM)	93 ± 7	101 ± 22
N144 (25 μM)	89 ± 9	104 ± 4
NEM (100 μM)	48 ± 7*	34 ± 2**

known to abolish the inside vesicular positive electrochemical potential. The Mg^{2+}-ATP-dependent uptakes of both GABA and glutamate were inhibited by thiocyanate in a dose-dependent manner (Fig. 1*d*). The IC_{50} values were calculated as 3.9 mM and 1.9 mM for uptake of GABA and glutamate respectively.

Vesicular uptake and Mg^{2+}-ATPase activity in a more purified synaptic-vesicle fraction

It has been reported (Diebler & Lazereg, 1985; Rothlein & Parsons, 1982) that the anion-transport blocker SITS also inhibits the Mg^{2+}-ATPase of *Torpedo*. We have therefore studied the effect of SITS on the vesicular Mg^{2+}-ATPase of rat brain. In these experiments we used a more purified synaptic vesicle fraction, i.e. synaptic vesicles chromatographed on a controlled-pore glass column. A typical run is shown in Fig. 2. The Mg^{2+}-ATPase was eluted in two peaks. Traces of AChE, a plasma-membrane marker, were co-eluted with the first peak. The uptake of GABA and glutamate was co-eluted with the second peak, which was almost free of AChE contamination. In the vesicle fraction, specific activity of the Mg^{2+}-ATPase was enriched by a factor 2, as compared with 3-fold enrichment in the uptake of GABA and glutamate.

To obtain enough material, fractions 13–17 (I) and 19–24 (II) were pooled. The effects of SITS and N144 on the Mg^{2+}-ATPase (Table 1) and the vesicular uptake (Table 2) were examined. In these experiments both the vesicular uptake and the ATPase were examined in 110 mM-potassium tartrate and 10 mM-Hepes, pH 7.4. The ATPase experiments were done in the presence of 50 μM-vanadate, 1 mM-ouabain and 5 μg of oligomycin B/ml. Ouabain and vanadate inhibit plasma-membrane ATPase, and oligomycin B is known to inhibit the mitochondrial H^+-ATPase. The Mg^{2+}-ATPase of fractions I and II was inhibited by 42 and 27% respectively by including the three inhibitors. N144 had almost no effect on the ATPase activity at all, neither fraction I nor II (Table 1). The uptake of glutamate into fraction II was inhibited by 78%, whereas the uptake of GABA was inhibited by 49%, by 25 μM-SITS (Table 2). The vesicular Mg^{2+}-ATPase activity was inhibited by 31% by 25 μM-SITS.

The vesicular Mg^{2+}-ATPase is known to be inhibited by *N*-ethylmaleimide (NEM) (Grønberg & Flatmark, 1987). In the presence of 100 μM-NEM the ATPase activity in fraction II was inhibited by 65%, whereas that in fraction I was inhibited by 45%. If SITS (25 μM) was added in the presence of NEM (100 μM) no further inhibition was seen.

Table 2. Effects of SITS and N144 on the uptake of GABA and glutamate into vesicles purified on a controlled-pore glass column

Fractions were pooled as described in the Results section and in Table 1. In the uptake experiments, only fraction II was used, owing to the low uptake activity in fraction I. The uptake was performed as described in the Experimental section. The results are expressed as means ± S.E.M. of three or four independent experiments: $^{*}P < 0.05$, $^{**}P < 0.005$ by Student's *t* test. The control values for fraction II were calculated as 6600 ± 600 and 1110 ± 160 pmol/min/mg of protein for the uptakes of glutamate and GABA respectively.

	Vesicular uptake [relative activity (%)]	
Inhibitor	Glutamate	GABA
SITS (1 μM)	85 ± 4*	111 ± 12
SITS (10 μM)	46 ± 6**	82 ± 12
SITS (25 μM)	22 ± 1**	51 ± 4**
N144 (1 μM)	91 ± 6	-
N144 (10 μM)	61 ± 9*	84 ± 13
N144 (25 μM)	31 ± 5*	55 ± 8*

Ion-sensitivity of the vesicular uptake of GABA and glutamate

Different anions were tested for their capacity either to stimulate or to inhibit the uptake of GABA and glutamate (Tables 3 and 4). The experiments were performed in the presence of 4 mM-$MgSO_4$, and different anions were successively added. Low concentrations of either KCl or KBr hardly affected the uptake of GABA (Table 3), whereas the uptake of glutamate was

Table 3. Effects of various anions on the vesicular uptake of GABA

A vesicle fraction (D-fraction) was incubated in 0.25 M-sucrose/10 mM-Tris/maleate (pH 7.4)/4 mM-$MgSO_4$/2 mM-ATP/1 mM-[^{3}H]GABA (2.9 mCi/mmol) for 3 min at 30 °C. The control value in the absence of any of the salts in the table was 830 ± 70 pmol/min per mg of protein (n = 6). Data (means ± S.E.M.) are percentages relative to the control (nos. of determinations in parentheses). The significance of differences was calculated by Student's t test: $^*P < 0.05$, $^{**}P < 0.005$

Salt added	Concn....	GABA uptake [relative activity (%)] 1 mM	5 mM	50 mM
KF		99 ± 4 (4)	95 ± 5 (4)	78 ± 7 (4)*
KCl		94 ± 5 (4)	91 ± 3 (5)	76 ± 3 (4)**
KBr		95 ± 5 (4)	90 ± 8 (4)	53 ± 4 (4)**
KI		95 ± 7 (4)	84 ± 7 (4)	24 ± 3 (4)**
KNO_3		71 ± 7 (4)*	36 ± 5 (4)**	12 ± 3 (4)**

Table 4. Effects of different anions on the vesicular uptake of glutamate

A vesicle fraction (D-fraction) was incubated in 0.25 M-sucrose, 10 mM-Tris/maleate (pH 7.4)/4 mM-$MgSO_4$/2 mM-ATP/1 mM-L-[^{3}H]glutamate (1.9 mCi/mmol) for 3 min at 30 °C. The control value was 560 ± 70 (n = 7) pmol/min per mg of protein. The uptake is expressed as percentage of control, and the data are means ± S.E.M. (nos. of determinations in parentheses): $^*P < 0.05$, $^{**}P < 0.005$ by Student's t test.

Salt added	Concn....	Glutamate uptake [relative uptake (%)] 1 mM	5 mM	50 mM
KF		93 ± 9 (5)	55 ± 3 (4)**	13 ± 7 (4)**
KCl		320 ± 50 (5)**	400 ± 70 (6)**	127 ± 14 (4)
KBr		410 ± 80 (6)**	450 ± 80 (5)**	54 ± 13 (4)*
KI		200 ± 40 (6)*	160 ± 40 (4)	11 ± 4 (4)**
KNO_3		180 ± 30 (6)*	118 ± 34 (4)	12 ± 2 (4)**

stimulated about 3- and 4-fold respectively (Table 4). Previously we have shown that K^+ had no effect on the uptake of GABA and glutamate (Fykse *et al.*, 1989). KF was a much more potent inhibitor of the glutamate uptake than of the GABA uptake. Low concentrations of KI and KNO_3 stimulated the uptake of glutamate significantly, whereas high concentrations inhibited both uptake systems.

To examine if SO_4^{2-} had any effect on the vesicular uptake, we used Mg^{2+}–ATP salt in the incubation mixture. Also in this case the uptake of GABA was not stimulated by addition of low concentrations of Cl^-, whereas the uptake of glutamate was highly stimulated, as before.

DISCUSSION

In the present study we have shown that the agents SITS, DIDS and N144 were about 10 times more potent in inhibiting the Mg^{2+}–ATP-dependent uptake of glutamate than the corresponding uptake of GABA into vesicles. The effect of SITS and N144 could not be explained by an effect on the vesicular Mg^{2+}-ATPase, since the Mg^{2+}-ATPase was less inhibited than the vesicular uptake of glutamate. In addition, we have extended the previous findings of Naito & Ueda (1985), Fykse *et al.* (1989) and Kish *et al.* (1989) that the vesicular uptakes of GABA and glutamate have different sensitivity to permeant anions. We have also shown that the inhibitory effect of SITS on the uptake of glutamate was less potent in the absence of Cl^-.

Since SITS and N144 inhibited the glutamate uptake more than hydrolysis of ATP, it is likely that their inhibitory effect was due to an effect on the glutamate carrier and not on the vesicle H^+-ATPase. This agrees with the general view that the vesicle H^+-ATPase is a well-preserved structure belonging to a class of enzyme (vacuolar type) responsible for acidification of different intracellular organelles (Nelson, 1986, 1987; Cidon & Sihra, 1989). The vesicle H^+-ATPase is immunologically related to the chromaffin-granule enzyme (Cidon & Sihra, 1989), and the mammalian vesicle H^+-ATPase closely resembled the vacuolar ATPase of chromaffin granules (Floor *et al.*, 1990). It is therefore reasonable to assume that SITS, DIDS and N144 did not primarily affect the Mg^{2+}-ATPase in the concentration range used in the present experiments.

The effect of SITS on the synaptosomal high-affinity uptake of glutamate was more than 100-fold less potent (Fosse *et al.*, 1989) than the effect on the vesicular uptake. This shows the large differences in the structure of the vesicular transporter and the high-affinity glutamate transporter of synaptosomes.

Agents which affect the Mg^{2+}-ATPase, NEM and *N,N'*-dicyclohexylcarbodi-imide ('DCCD'), or the electrochemical proton gradient, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), nigericin and gramicidin seems to inhibit the uptake of GABA and glutamate more similar than SITS, DIDS and N144 (Fykse *et al.*, 1989). The anion SCN^- have been shown to inhibit the uptake of glutamate and GABA (Shioi *et al.*, 1989; Hell *et al.*, 1990), and in the present work the uptake of GABA and glutamate was inhibited nearly to the same extent by SCN^-.

The point then arises whether SITS, DIDS and N144 may affect the glutamate transporter. The three compounds, although only to a small extent N144, have two negatively charged groups, as in glutamate. The distance between the groups (C_4) is, however, larger than in glutamate, and should not give inhibition (Christensen *et al.*, 1991). In other biological systems such as red blood cells, the stilbenedisulphonate derivatives SITS and DIDS are known to be blockers of anion transport (Cabantchik *et al.*, 1978), and Cabantchik *et al.* (1978) have shown that 5–8 μM-DIDS almost completely inhibited anion exchange in red blood cells. This is in agreement with the effect of DIDS on the uptake of glutamate. In the absence of Cl^-, the effect of SITS on the uptake of glutamate was less potent. These results indicate that a Cl^- channel or a Cl^--binding site might be involved in the uptake of glutamate. This is in agreement with the fact that different anions affected the uptake of GABA and glutamate differently. Low concentrations of several permeant anions such as Cl^-, Br^- and I^- stimulated the uptake of glutamate, which are in agreement with Naito & Ueda (1985). Low concentrations of F^- inhibited the uptake of glutamate significantly. In contrast, low concentrations of permeant anions did not stimulate the uptake of GABA in the preparations, which is consistent with the effect of Cl^- on the uptake of GABA (Fykse *et al.*, 1989; Kish *et al.*, 1989). In contrast, Hell *et al.* (1990) found a 40% decrease in the GABA uptake in the absence of Cl^-; maximal uptake was observed in the range of 4–50 mM-Cl^- ions. Even in the experiments where we added Mg^{2+} as Mg^{2+}–ATP to avoid SO_4^{2-} in the incubation mixture, we did not find any stimulation of the GABA uptake by Cl^-. The uptake of glutamate was highly stimulated as before. All results indicate at least a different anion-sensitivity of the uptake systems for GABA and glutamate.

We also tested the more specific anion-channel blocker N144 (Wangemann *et al.*, 1986), since some of the present results support the idea that a Cl^- channel might be involved in the uptake of glutamate. The effects on the uptake of GABA and glutamate were in agreement with the effects of SITS and DIDS.

Dayanithi & Nordmann (1989) found that N144 caused release of vasopressin from rat permeabilized neurohypophysial nerve endings, and they concluded that a Cl^- channel was involved in the release process. They found the IC_{50} value to be 5 μM. Release of adrenaline from chromaffin granules has also been shown to be inhibited by SITS, and anions such as Cl^- were required for the release process (Pazoles & Pollard, 1978). Cidon & Nelson (1982) investigated the effect of SITS on the Mg^{2+}-ATPase of chromaffin granules and concluded that up to a concentration of 10 μM-SITS the inhibition was due to a blockage of a Cl^- channel.

To explain the different effects of SITS, DIDS and N144 on the neurotransmitter uptakes, a direct effect on at least part of the glutamate transporter seems to be a reasonable explanation. The effects of low concentrations of permeant anions on the uptake of glutamate, and the more potent effect of anion-channel blockers, indicate involvement of an anion channel or an anion site. The lack of effect of these anions on the uptake of GABA, and the less potent effect of the anion-channel blockers, indicate that permeant anions are not directly involved in the uptake of GABA. One possible mechanism for the uptake of glutamate is that a glutamate/Cl^- antiport exists. More experiments need to be done to show clearly whether a Cl^- channel, a Cl^--binding site and a glutamate/Cl^- antiport are involved in the uptake of glutamate.

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PAPER IV

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Regional distribution of γ-aminobutyrate and L-glutamate uptake into synaptic vesicles isolated from rat brain

Else M. Fykse and Frode Fonnum

Norwegian Defence Research Establishment, Division for Environmental Toxicology, Kjeller (Norway)

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Key words: Synaptic vesicle; Vesicular uptake; γ-Aminobutyric acid; L-Glutamate; Regional distribution

The ATP-dependent uptake of GABA and L-glutamate into synaptic vesicles isolated from 4 different regions of the rat brain was studied. The regional distribution of the vesicular uptake was related to the Na^+-dependent synaptosomal uptake, which, as a first approximation, corresponds to the distribution of GABAergic and glutamatergic terminals. The ratio found between the vesicular GABA and L-glutamate uptake varied between 1.3 and 6.2. This indicates that GABA and L-glutamate are taken up into different vesicle populations.

There is now substantial evidence that the amino acid neurotransmitters γ-aminobutyrate (GABA) and L-glutamate are specifically taken up into mammalian brain synaptic vesicles [1, 6, 9, 10, 12]. The uptake is highly dependent on Mg^{2+}, ATP and a transmembrane pH gradient. The vesicular uptake differs from glial and synaptosomal uptake in that it is not inhibited by inhibitors of these uptakes, and that it is not stimulated by high concentrations of NaCl [6, 9, 10]. GABA uptake is more labile than the L-glutamate uptake, and has been difficult to demonstrate. Thus in an attempt to isolate vesicles by immunoprecipitation, the uptake of GABA was lost [9]. Subcellular fractionation based on hypo-osmotic shock of synaptosomes results in vesicles with stable GABA uptake [6–8].

Previous studies on vesicular uptake have mostly dealt with the uptake into synaptic vesicles isolated from the whole brain [1, 6, 7, 9, 10]. In the present study we have compared the uptake of GABA and L-glutamate into synaptic vesicles and synaptosomes isolated from different brain regions. The object was to see if the regional distribution of the vesicular uptake of L-glutamate and GABA differ, and if the vesicular uptake is correlated with the distribution of GABAergic and glutamatergic terminals. Such findings would also indicate that the two uptake processes are specific.

Correspondence: E.M. Fykse, Norwegian Defence Research Establishment, Division for Environmental Toxicology, P.O. Box 25, N-2007 Kjeller, Norway.

Brains from 10 male Wistar rats weighing 200–250 g were removed after decapitation. The brains were dissected into 4 regions, cerebral cortex, cerebellum, medulla and subcortical telencephalon (i.e. forebrain after removal of cortex). The vesicular uptake is low and requires a large amount of material, preventing a separation of the brain into several other regions at the present time. A 10% homogenate was made in 0.32 M sucrose, 10 mM Tris-maleate (pH 7.4) and 1.0 mM EGTA. After centrifugation of the homogenate at 1000 *g* for 10 min, the supernatant was made 0.8 M in sucrose and centrifuged at 20,000 *g* for 30 min. This allows the separation of myelin and microsomes from the synaptosomes. The crude synaptosomal pellet was osmotically shocked by resuspension in 10 mM Tris-maleate (pH 7.4). After 10 min, sucrose was added to 0.3 M, and the solution was centrifuged at 17,000 *g* for 30 min. The supernatant, enriched in synaptic vesicles [13], was used for vesicular uptake studies. The variation in vesicular proteins per gram original tissue of each brain region was not significant. The average protein contents were 1.10, 0.90, 1.00 and 1.10 mg protein per gram original tissue in cerebral cortex, cerebellum, medulla and the subcortical telencephalon respectively. The crude synaptosomal fraction was subjected to high affinity uptake of GABA and L-glutamate.

Vesicular uptake of [^{3}H]GABA and L-[^{3}H]glutamate was determined as described by Fykse and Fonnum [6]. Synaptic vesicles (100–160 μg protein) were incubated with 1 mM [^{3}H]GABA or L-[^{3}H]glutamate (5 mCi/mmol), 2 mM ATP (disodium salt neutralized with Tris base) and 4 mM $MgCl_2$. The vesicles were preincubated at 30°C for 15 min before incubation with ATP and tritiated neurotransmitter for 3 min at 30°C. The reaction was stopped by filtration through a Millipore HAWP filter (24 mm, 0.45 μm), and the radioactivity was determined in a Pachard Tri Carb 2200 Liquid scintillation counter with a counting efficiency of 54–58%. Blanks were treated similarly, but were incubated at 0°C. The blank values were 548 ± 18 cpm ($n = 64$) for GABA and 516 ± 17 cpm ($n = 62$) for L-glutamate. The blank values corresponded to about 55% and 30% of the radioactivity retained on the filters for the GABA and L-glutamate uptake respectively. The blank values did not vary significantly among the different brain areas.

High affinity uptake of GABA and L-glutamate into synaptosomes was measured as described by Fonnum et al. [5]. Two μl crude synaptosomal fractions (containing 3–5 μg protein) were added to 0.5 ml Tris-Krebs medium containing (mM): Tris 15, NaCl 140, KCl 5, $CaCl_2$ 1.2, $MgSO_4$ 1.2, Na_2HPO_4 1.2, glucose 10, pH adjusted to 7.4. The mixtures were preincubated for 15 min before incubation with 75–80 nM tritiated transmitter (20–30 Ci/mmol) for 3 min at 25°C. The uptake was terminated by filtration in a Skatron cell-harvester with a glassfiber filtermat.

In this study we have investigated the uptake of GABA and L-glutamate into a crude vesicle fraction isolated from different brain areas (Table I). We have confirmed that with this procedure the uptake was highly dependent on ATP and independent of Na^+ ions, showing the absence of plasma membrane uptake systems [6]. Separate experiments showed that the uptake was linear up to 3 min and linear with protein concentration.

In order to investigate if the distribution of vesicular uptake corresponded with

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TABLE I

UPTAKE OF GABA AND L-GLUTAMATE INTO SYNAPTIC VESICLES ISOLATED FROM DIFFERENT BRAIN AREAS

A crude vesicle fraction was incubated in 0.32 M sucrose, 10 mM Tris-maleate (pH 7.4), 4 mM $MgCl_2$, 2 mM Na_2ATP and 1 mM L-[^{3}H]glutamate or [^{3}H]GABA (5 mCi/mmol) for 3 min at 30°C. Data are means ± S.E.M. from 6 different experiments.

Region	Vesicular uptake (pmol/min/g original tissue)		
	GABA	L-Glutamate	Ratio L-Glu/GABA
Cerebral cortex	63 ± 11	388 ± 58	6.2
Cerebellum	48 ± 13	186 ± 25	3.9
Medulla	144 ± 31	188 ± 43	1.3
Subcortical telencephalon	216 ± 35	459 ± 55	2.1

the distribution of glutamatergic and GABAergic terminals, we studied the sodium-dependent synaptosomal uptake into the same regions (Table II). The ratio between GABA and L-glutamate uptake into synaptic vesicles corresponded well with the ratio between GABA and L-glutamate uptake into synaptosomes from the same regions. The uptake of L-glutamate into synaptic vesicles was highest in the cerebral cortex and also in the subcortical telencephalon containing among others striatal and thalamic regions. These are regions known to be rich in glutamatergic terminals [3, 11]. The uptake was lower in cerebellum and medulla. The regional distribution of the vesicular uptake of L-glutamate was in agreement with the synaptosomal uptake of L-glutamate, except that the synaptosomal uptake in cerebral cortex was twice the uptake in the subcortical telencephalon. The area which showed the highest vesicular

TABLE II

UPTAKE OF GABA AND L-GLUTAMATE INTO A CRUDE SYNAPTOSOMAL FRACTION ISOLATED FROM DIFFERENT BRAIN AREAS

A crude synaptosomal fraction was incubated in Krebs solution and 75 nM [^{3}H]GABA (24 Ci/mmol) or 80 nM L-[^{3}H]glutamate (25 Ci/mmol) at 25°C for 3 min. Data are means ± S.E.M. from 5 different experiments.

Region	Synaptosomal uptake (pmol/min/g original tissue)		
	GABA	L-Glutamate	Ratio L-Glu/GABA
Cerebral cortex	344 ± 47	1852 ± 99	5.4
Cerebellum	107 ± 29	468 ± 58	4.4
Medulla	122 ± 14	245 ± 26	2.0
Subcortical telencephalon	344 ± 64	934 ± 64	2.7

uptake of GABA, was the subcortical telencephalon. This corresponded well with the distribution of the synaptosomal GABA uptake. The subcortical telencephalon contains among others hypothalamus, globus pallidus and substantia nigra, which are regions known to be rich in GABAergic neurons [4, 11].

As previously described, the vesicular GABA uptake is not inhibited by L-glutamate, and the vesicular L-glutamate uptake is not inhibited by GABA [6, 10]. This indicates that GABA and L-glutamate are taken up at different sites of synaptic vesicles. In this study we found a different ratio between L-glutamate and GABA uptake into different brain regions. This supports the notion that GABA and L-glutamate are taken up into different vesicle populations. Recently, ATP-dependent L-glutamate uptake in the cerebellar synaptic vesicle fraction was reduced by about 60% in mice lacking granule cells, but not in mice lacking Purkinje cells [2]. These results point in the same direction as ours.

The vesicular uptake ratio L-glutamate/GABA in cerebral cortex was 6.2, which indicates that the GABA uptake is 16% compared to the L-glutamate uptake. In medulla the ratio L-glutamate/GABA was only 1.3, which means that the uptake of GABA is 77% compared to the L-glutamate uptake. The ratio found between GABA and L-glutamate uptake in medulla is the highest ever reported.

We therefore conclude that the vesicular uptake of GABA and L-glutamate corresponds with the expected distribution of GABAergic and glutamatergic terminals. These results support the idea that GABA and L-glutamate are taken up into different populations of synaptic vesicles. At present, our working hypothesis is that the vesicular uptake is the key factor in differentiating between different amino acids as transmitter candidates in specific terminals.

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PAPER V

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Inhibition of γ-aminobutyrate and glycine uptake into synaptic vesicles

Hege Christensen, Else M. Fykse and Frode Fonnum

Norwegian Defence Research Establishment, Division for Environmental Toxicology, N-2007 Kjeller, Norway

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The substrate specificity of vesicular GABA and glycine uptake was studied in vesicle fractions from brain and spinal cord, respectively. Glycine, β-alanine and γ-vinyl-GABA were competitive inhibitors of the GABA uptake by synaptic vesicles in brain. Likewise GABA and β-alanine turned out to be competitive inhibitors of vesicular uptake of glycine in spinal cord. The apparent K_i values were in the same range as the respective K_m values for the transport systems. Accumulation of different amino acids were examined, and the structurally related amino acids GABA, β-alanine and glycine were all taken up by both vesicle fractions. In the present study, we suggest that there are similarities in the vesicular transporters for GABA and glycine, and the two amino acids are probably taken up into the same vesicle population. The key factor in differentiating between GABA and glycine as transmitters in the terminals could be the synthesis and the high-affinity synaptosomal uptake.

Synaptic vesicles; Glycine; GABA; Vesicular uptake

1. Introduction

γ-Aminobutyrate (GABA) and glycine are regarded as the major inhibitory neurotransmitters in the vertebrate central nervous system (CNS). GABA is a transmitter in both the upper and lower part of the CNS, whereas glycine is an established transmitter in the lower part of the CNS (Aprison and Nadi, 1978; Fonnum, 1987). The high-affinity uptake of the two amino acids over plasma membranes differs between different regions of CNS (Johnston and Iversen, 1971). Furthermore, the plasma membrane uptake of GABA is reported not to be inhibited by glycine and vice versa (Balcar and Johnston, 1973). Postsynaptically both neurotransmitters lead to inhibition by an opening of chloride channels. The receptors for the two amino acids show sequence homology (Barnard et al., 1987).

ATP-dependent GABA and glycine uptake in brain and spinal cord synaptic vesicles, respectively, has recently been demonstrated (Christensen et al., 1990; Fykse and Fonnum, 1988; Hell et al., 1988; Kish et al., 1989). The accumulation of the two inhibitory transmitters is driven by an electrochemical proton gradient generated by a similar Mg^{2+}-ATPase. The uptakes are not stimulated by Na^+ and appear to be different from that of glial cells and synaptosomes (Christensen et al., 1990; Fykse and Fonnum, 1988).

Kish et al. (1989) have recently suggested that uptake of GABA and glycine into synaptic vesicles are distinct from each other. We have recently shown that a high concentration of GABA and β-alanine inhibited the uptake of glycine into a vesicle fraction from spinal cord (Christensen et al., 1990). In the present study we have therefore examined the substrate specificity of the vesicular transporters of GABA and glycine in brain and spinal cord, respectively. The uptake of β-alanine, taurine and aspartate into vesicles has also been examined.

Correspondence to H. Christensen, Norwegian Defence Research Establishment, Division for Environmental Toxicology, P.O. Box 25, N-2007 Kjeller, Norway.

2. Materials and methods

2.1. Materials

GABA, L-glutamate (dipotassium salt), L-aspartate (disodium salt), glycine, taurine, β-alanine, L-alanine, L-serine, N-methylalanine, N-methylglycine (sarcosine), allylglycine, guanidino-propionate, γ-vinyl-GABA, nipecotate, diaminobutyrate (DABA), strychnine and ATP (disodium salt) were purchased from Sigma Chemical Co. St. Louis, MO, U.S.A. [3-^{3}H(N)]β-alanine (120 Ci/mmol), [2,3-^{3}H]GABA (40 Ci/mmol), [^{3}H]glycine (53.3 Ci/mmol), L-[2,3-^{3}H]aspartate (14.9 Ci/mmol), L-[2,3-^{3}H]glutamate (25 Ci/mmol) and [2-^{3}H(N)]taurine (20.1 Ci/mmol) were from New England Nuclear.

2.2. Preparation of synaptic vesicles

In each experiment 10 male Wistar rats (200-250 g) were killed by decapitation, and the brains and the spinal cords were quickly removed. Synaptic vesicles were prepared as described by Fykse and Fonnum (1988) and Christensen et al. (1990). Homogenates (10%) from both brain and spinal cord were made in 0.32 M sucrose, 10 mM Tris-maleate (pH 7.4) and 1.0 mM EGTA. The homogenates were centrifuged 10 min at $800 \times g$, and the supernatants were centrifuged at $15,500 \times g$ for 30 min to obtain P_2 fractions (crude synaptosomal fractions). The spinal cord supernatant was made 0.8 M in sucrose before centrifugation, to remove myelin and microsomes from the P_2 fraction. The crude synaptosomal fraction from both brain and spinal cord were osmotically shocked by resuspension in 10 mM Tris-maleate (pH 7.4) and 0.1 mM EGTA, and the solutions were centrifuged at $13,000 \times g$ for 30 min. In the spinal cord experiments this supernatant was made 0.30 M in sucrose and used in the vesicular uptake studies. The supernatant from the brain preparation was laid on top of 0.4 M and 0.6 M sucrose solutions and centrifuged in a Contron TST 28.38 rotor at $65,000 \times g$ for 2 h. The vesicle fraction was isolated from the band containing 0.4 M sucrose (D-fraction). The vesicle preparations were stored in liquid nitrogen without loss of activity. The vesicular uptakes were not contaminated by plasma membrane uptake, since they were not stimulated by Na^+ (Christensen et al., 1990; Fykse and Fonnum, 1988; Fykse et al., 1989). Furthermore the uptakes were dependent upon Mg^{2+} and ATP and inhibited by proton ionophores.

2.3. Assay for vesicular GABA and glycine uptake

Uptake of GABA into brain synaptic vesicles and glycine into spinal cord vesicles was assayed as previously described (Christensen et al., 1990; Fykse and Fonnum, 1988). Synaptic vesicles (0.1-0.2 mg of protein) were preincubated for 15 min at 30°C in 0.30 M sucrose, 10 mM Tris-maleate (pH 7.4), 4 mM $MgCl_2$ and the test agent. The vesicles were incubated for 1.5 min (brain) or 2 min (spinal cord) with 25 μl of a substrate solution containing ATP (final concentration = 2 mM) and [^{3}H]GABA (final concentration = 1 mM; 1 μCi) or [^{3}H]glycine (final concentration 1 mM; 1.5 μCi). Uptake was terminated by addition of 7 ml of ice-cold 0.15 M KCl immediately followed by rapid filtration through a millipore HAWP filter (diameter 25 mm, pore size 0.45 μm). The incubation tubes and the filters were washed twice with 0.15 M KCl solution. The filters were dissolved in 10 ml of Filter Count, and the radioactivity was determined in a Packard Tri-Carb 2200 liquid scintillation counter with a counting efficiency of 50-56%. Blanks were treated in the same way but incubated at 0°C. Blank values did not vary significantly under the different treatments. The blank values for the uptake of different amino acids into brain vesicles were: GABA, 374 ± 14 cpm; glycine, 396 ± 30 cpm; β-alanine, 418 ± 17 cpm; glutamate, 205 ± 23 cpm. The blank values for the uptake into spinal cord synaptic vesicles were: GABA, 410 ± 45 cpm; glycine, 363 ± 12 cpm; β-alanine, 376 ± 61 cpm; glutamate, 402 ± 44 cpm. The values corresponded to about 10-30% of the radioactivities retained on the filters, but binding of the substrate to the filters accounted for 70% of the blank values. Assays were carried out in duplicate. The test agents were added in the preincubation mixture. Strychnine was dissolved in absolute ethanol (1.5%), and ethanol in this concentration inhibited the vesicular uptake of glycine by 11%. The uptake of [^{3}H]taurine (1.5 μCi), [^{3}H]β-alanine (1.5 μCi), [^{3}H]L-aspartate (1.5 μCi) and [^{3}H]L-glutamate (0.5 μCi) was assayed in the same way in the presence of 1 mM of the respective amino acids, 2 mM ATP and 4 mM $MgCl_2$. In the spinal cord uptake experiments 1.5 μCi of [^{3}H]L-glutamate and [^{3}H]GABA were used. The uptake was linear over the protein ranges used for both brain and spinal cord vesicles. Protein was determined according to Lowry et al. (1951) with bovine serum albumin as standard.

2.4. Calculations and statistics

Results are expressed as mean ± S.E. of absolute or relative uptake (as percent of controls). Groups of data

TABLE 1

Effects of different agents on the vesicular uptake of GABA into synaptic vesicles isolated from brain. The vesicle fraction was incubated in 0.30 M sucrose, 10 mM Tris-maleate (pH 7.4), 2 mM ATP, 4 mM $MgCl_2$ and 1 mM [^{3}H]GABA (1 μCi) at 30°C for 1.5 min. The different test agents, in 5 and 10 mM concentrations, were added to the preincubation mixture. The control value for the vesicular uptake of GABA was 954 ± 82 pmol/min/mg of protein (n = 10). Data are expressed in percent of control (mean ± S.E. for the number of determinations given in parentheses).

Test agents	GABA uptake (%)	
	5 mM	10 mM
DABA	84 ± 9 (5)	71 ± 11 (5)
Nipecotate	88 ± 7 (5)	81 ± 4 (5) [a]
L-Glutamate	105 ± 7 (5)	105 ± 5 (5)
L-Aspartate	97 ± 5 (5)	84 ± 3 (5) [a]
Guanidino-propionate	95 ± 4 (3)	77 ± 7 (3)
Allylglycine	90 ± 9 (4)	68 ± 5 (3) [a]
GVG	39 ± 5 (6) [b]	27 ± 4 (5) [b]
N-Methylglycine		91 ± 3 (3)
N-Methylalanine		97 ± 3 (3)
L-Serine		114 ± 10 (3)
Taurine		109 ± 8 (3)
L-Alanine		73 ± 6 (5)
Glycine	54 ± 9 (4) [a]	34 ± 9 (4) [b]
β-Alanine	60 ± 7 (4) [a]	39 ± 9 (3) [a]
GABA		22 ± 2 (3) [b]

[a] $P < 0.01$, [b] $P < 0.001$, by Student's t test.

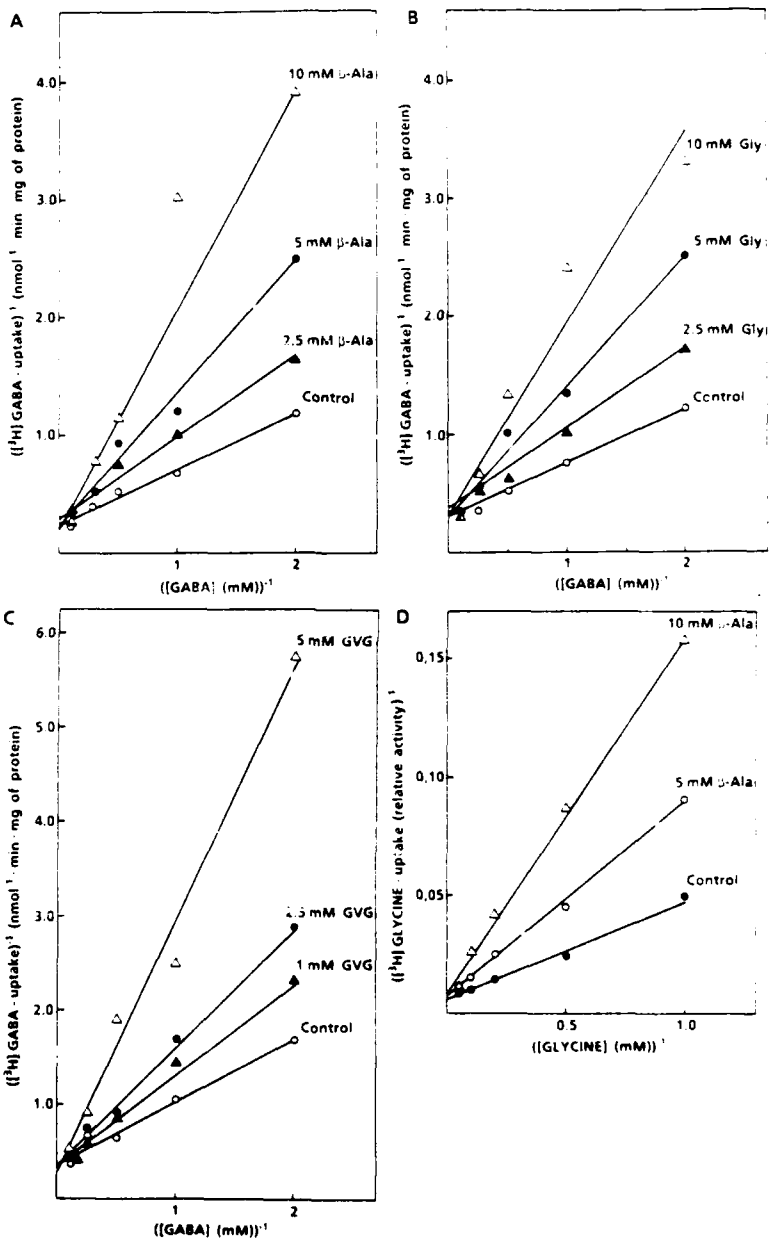

Fig. 1. Double-reciprocal plots of the vesicular uptake of GABA or glycine against the concentration of GABA or glycine in the absence and in the presence of different structural analogues. A, B and C show the uptake of GABA into synaptic vesicles from brain. D shows the vesicular uptake of glycine into a spinal cord preparation. The different inhibitors added were (A) β-alanine, (B) glycine, (C) GVG, and (D) β-alanine. The apparent K_i values for the GABA uptake were calculated to be 6.6 mM for β-alanine, 3.7 mM for glycine and 1.8 mM for GVG. The apparent K_i value for the glycine uptake was 3.7 mM for β-alanine. Synaptic vesicles isolated from brain or spinal cord were incubated as described in Materials and methods. In these kinetic experiments the concentration of GABA was varied between 0.5 and 10 mM, and the concentration of glycine was varied between 1 and 20 mM. Each point represents the average of at least three separate experiments.

were analyzed by Student's t test. All figures show mean values from at least three different independent experiments. Linear regression analysis was performed.

3. Results

3.1. Substrate specificity for GABA uptake into brain synaptic vesicles

In order to examine the specificity of the vesicular GABA translocator, different GABA and glycine analogues were tested for their ability to inhibit the uptake of GABA (table 1). Diaminobutyrate (DABA) and nipecotate, agents known to inhibit the sodium-dependent GABA uptake by plasma membranes (Krogsgaard-Larsen and Johnston, 1975), had almost no effect on the vesicular uptake of GABA. The GABA analogues, guanidino-propionate and allylglycine had only a small effect on the uptake of GABA. In contrast, γ-vinyl-GABA (GVG) had a very potent inhibitory effect. The effect of GVG on the vesicular uptake of GABA appeared to be competitive (fig. 1). The apparent K_i value was calculated to be 1.8 mM.

The structural analogues N-methylglycine and N-methylalanine did not inhibit the vesicular uptake (table 1). In addition, the amino acids L-serine and taurine had no inhibitory effect either. L-alanine inhibited the uptake by 27%. The acidic amino acids L-glutamate and L-aspartate at 5 and 10 mM did not affect the uptake of GABA.

The amino acids glycine and β-alanine were potent inhibitors of the uptake of GABA (table 1), and the inhibition turned out to be competitive (fig. 1). Apparent K_i values were calculated to be 3.7 and 6.6 mM, respectively.

3.2. Substrate specificity for glycine uptake into spinal cord synaptic vesicles

To examine the specificity of the glycine translocator in spinal cord, some GABA and glycine analogues were used. Addition of DABA had little effect on vesicular glycine uptake, and nipecotate inhibited uptake by 36% (table 2). The GABA analogue allylglycine had only a small effect on the uptake, while the branched chain amino acid GVG was a potent inhibitor of the glycine transport. The glycine receptor antagonist strychnine caused considerable inhibition when present in a high concentration (5 mM).

The effect of various structurally related amino acids on the uptake of glycine into synaptic vesicles from spinal cord was examined. Table 2 shows that addition of a high concentration of the N-substituted amino acids N-methylalanine or N-methylglycine did not affect uptake of glycine. The amino acids L-serine and taurine had no effect either. However, vesicular glycine uptake was highly inhibited by the structurally related amino acids GABA and β-alanine. L-Alanine was less potent than the others.

Further investigations indicated a competitive inhibition by β-alanine on the uptake of glycine (fig. 1). The apparent K_i value was calculated to be 3.7 mM. The effect of GABA on vesicular uptake of glycine also seems to be competitive, but due to high inhibition the

TABLE 2

Effects of different agents on the vesicular uptake of glycine into synaptic vesicles isolated from spinal cord. The vesicle fraction was incubated in 0.30 M sucrose, 10 mM Tris-maleate, 4 mM $MgCl_2$, 2 mM ATP and 1 mM [^{3}H]glycine (1.5 μCi). The different test agents, in 5 and 10 mM concentrations, were added to the preincubation mixture. The test tubes were incubated at 30 °C for 2 min. The control value in the absence of test agents was 402 ± 23 pmol/min/mg of protein (n = 15). Data are presented as mean ± S.E. of relative uptakes, expressed in percent of control in each experiment. The number of determinations is given in parentheses.

Test agents	Glycine uptake (%)	
	5 mM	10 mM
DABA		81 ± 2 (3) [a]
Nipecotate		64 ± 5 (4) [a]
Strychnine	11 ± 4 (4) [a]	
Allylglycine		80 ± 6 (5)
GVG	42 ± 7 (6) [a]	
N-Methylglycine		118 ± 16 (3)
N-Methylalanine		108 ± 4 (3)
L-Serine		92 ± 10 (3)
Taurine		93 ± 3 (3)
L-Alanine		55 ± 5 (4) [a]
GABA	34 ± 4 (5) [a]	25 ± 4 (5) [a]
β-Alanine	52 ± 3 (4) [a]	30 ± 5 (4) [a]
Glycine		45 ± 3 (3) [a]

[a] $P < 0.001$, by Student's t test.

TABLE 3

Vesicular uptake of different amino acids. Synaptic vesicles from rat brain and rat spinal cord were prepared and incubated with radioactive amino acids (concentration = 1 mM) as described in Materials and methods. The spinal cord vesicles were incubated for 2 min, while the brain vesicles were incubated for 1.5 min. All incubation mixtures contained 2 mM ATP and 4 mM $MgCl_2$ and were incubated at 30°C. Blanks were treated the same way but incubated at 0°C. Data are mean ± S.E. values. The number of determinations are given in parentheses.

Amino acid	Vesicular uptake (pmol/min/mg of protein)	
	Spinal cord	Brain
L-Glutamate	686 ± 57 (6)	2804 ± 268 (4)
GABA	764 ± 78 (5)	954 ± 82 (10)
Glycine	402 ± 23 (15)	488 ± 79 (3)
β-Alanine	261 ± 26 (3)	431 ± 83 (4)
L-Aspartate	N.D. [a] (3)	N.D. [a] (3)
Taurine	N.D. [a] (3)	N.D. [a] (3)

[a] Not detected

K_i value for GABA was estimated to be 3 mM. GABA turned out to be a slightly better inhibitor of the glycine uptake than β-alanine (table 2).

3.3. Uptake of different amino acids

Due to the ability of the structural analogues glycine, β-alanine, GABA and GVG to compete with vesicular uptake of GABA and glycine, we found it interesting to compare the uptake of radioactive glycine, β-alanine, GABA, L-glutamate, L-aspartate and taurine into synaptic vesicles from both brain and spinal cord (table 3). The uptake of glycine and β-alanine into brain vesicles was almost the same and constituted about 50% of the GABA uptake. Extract from vesicles subjected to β-alanine uptake was analyzed by HPLC, and at least 70% of the radioactivity travelled with the β-alanine peak. Previously the same has been shown for GABA and glycine uptake (Christensen et al., 1990; Fykse and Fonnum, 1988). Taurine and L-aspartate were not taken up by the vesicle fraction. Uptake of glutamate was also investigated, and it was found to be about three-fold higher than the GABA uptake. In the spinal cord experiments, however, the uptake of glutamate and GABA were similar, and they were found to be higher than the uptake of glycine and β-alanine. Aspartate and taurine were not accumulated at all. The uptake of β-alanine into both vesicle fractions was totally abolished when ATP was removed from the incubation mixture (results not shown).

4. Discussion

We have examined the substrate specificity of the uptake of GABA and glycine into rat brain and rat spinal cord synaptic vesicles, respectively. Uptake of GABA was mainly studied in synaptic vesicles isolated from rat brain by sucrose gradient centrifugation. Uptake of glycine was studied in synaptic vesicles isolated from spinal cord. The spinal cord synaptic vesicles were less pure than the brain vesicles (no gradient), but the myelin and membrane contamination were removed during the isolation. Synaptic vesicles from spinal cord were also isolated by sucrose gradient, and the fraction which contained vesicles showed similar properties (results not shown).

The present study showed that GABA competitively inhibited the uptake of glycine, and glycine was a competitive inhibitor of GABA uptake. Kish et al. (1989) did not find any significant inhibition of GABA and glycine on the vesicular uptake of glycine and GABA, respectively. They suggested therefore that the uptake of the two amino acids were different. They did not find an inhibition of the GABA and glycine uptake by other structural analogues either. We have repeated the inhibition experiments carried out by Kish et al. (1989) using 150 μM GABA and glycine and 10 min incubation time. At 10 min the two uptake systems are saturated. Under these conditions, which are not kinetically satisfactory, GABA and glycine at 5 mM did not inhibit the uptake of glycine and GABA, respectively (results not shown). Previously we did not find any effect of 10 mM β-alanine on the vesicular uptake of a low concentration (44 μM) of GABA into rat brain synaptic vesicles (Fykse and Fonnum, 1988). The lack of inhibition in these experiments was probably due to the different kinetic conditions used (Fykse and Fonnum, 1988; Kish et al., 1989). The concentrations were far below the apparent K_m values, which are about 6 and 8 mM for GABA and glycine uptake, respectively (Christensen et al., 1990; Fykse and Fonnum, 1988; Kish et al., 1989).

The uptake of GABA into brain vesicles was competitively inhibited by both β-alanine, glycine and GVG. Likewise the uptake of glycine into spinal cord synaptic vesicles was found to be inhibited by β-alanine, GABA and GVG. GABA, β-alanine and glycine show great structural similarities. The inhibitory constants (K_i) were found to be in the same range as the Michaelis-Menten constants (K_m) for the uptake of GABA and glycine (Christensen et al., 1990; Fykse and Fonnum, 1988; Kish et al., 1989). In contrast, glutamate had no effect on the uptake of GABA by synaptic vesicles from brain. Earlier we have reported that the vesicular uptake of glycine in spinal cord was not affected by glutamate (Christensen et al., 1990), which is in agreement with Kish et al. (1989).

Since β-alanine, glycine and GABA are competitive inhibitors of vesicular uptake, with K_i values in the same range as the respective K_m values, it was interesting to see if these agents were taken up into synaptic vesicles isolated from brain and spinal cord separately. The uptake of β-alanine and glycine into synaptic vesicles from brain was similar, but only about half of the uptake of GABA. Synaptic vesicles isolated from spinal cord were able to accumulate β-alanine, GABA and glutamate in addition to glycine. Taurine and L-aspartate were not taken up into synaptic vesicles neither from brain nor from spinal cord. These results indicate that the structural analogues β-alanine, glycine and GABA all acted as substrates in both vesicle preparations, and the amino acids were therefore probably taken up into the same vesicle population. On the other hand, glutamate and GABA have been shown to be taken up into different vesicle populations isolated from different brain regions (Fykse and Fonnum, 1989). Aspartate, which has been suggested to be a transmitter in spinal cord interneurons (Davidoff et al., 1967), was not taken up into synaptic vesicles from brain or spinal cord. The fact that aspartate was not taken up by the vesicles is in agreement with the results of Naito and

Ueda (1983). These results have severely weakened the position of aspartate as a transmitter. The results of Nicholls (1989) that endogenous aspartate is not released in a Ca^{2+}-dependent manner from cortical synaptosomes, also speak strongly against aspartate as a neurotransmitter in the CNS. In vivo Ca^{2+}-dependent release of aspartate from striatum has been found (Paulsen and Fonnum, 1989). Several pathways which could use aspartate instead of glutamate have been discussed (Fonnum, 1984). This raises the question that in vivo and also in slices it is possible that an exchange and interconversion between glutamate and aspartate may occur during the release experiments (Fonnum, 1991).

The N-methylated forms of the amino acids glycine and alanine were not able to inhibit the vesicular uptake of GABA or glycine, indicating that a primary amino group is an essential component of vesicular uptake of inhibitory amino acids. This is in agreement with the results of Kish et al. (1989). GVG was a better inhibitor than allylglycine, which only slightly inhibited the uptake. DABA and nipecotate are reported to be excellent inhibitors of the high-affinity uptake of GABA (Krogsgaard-Larsen and Johnston, 1975), but the two inhibitors had only a small effect on the vesicular uptake of GABA and glycine, which has been reported earlier, but different kinetic conditions were used in those experiments (Fykse and Fonnum, 1988; Kish et al., 1989).

These results indicate that the vesicular uptake of GABA into synaptic vesicles from brain (including cerebral cortex) and the vesicular uptake of glycine into synaptic vesicles from spinal cord are similar, and that the amino acids are probably taken up into the same vesicle population. In contrast, the high-affinity transport systems for glycine and GABA over plasma membranes are specific (Balcar and Johnston, 1973). A high-affinity plasma membrane uptake has been demonstrated for glycine in microslices from spinal cord, medulla and pons (Johnston and Iversen, 1971), although recently such uptake has also been described in a few supra-spinal regions (Debler and Lajtha, 1987; Gundlach and Beart, 1982; Wilkin et al., 1981). The high glycine/GABA uptake ratio in synaptic vesicles from whole brain (0.5) is unexpected in view of the limited distribution of high-affinity synaptosomal uptake of glycine. The uptake of glycine in brain vesicles is interesting, since glycine has a modulating effect on the NMDA receptor (Johnson and Ascher, 1987). Our finding is also interesting in view of the described colocalization of GABA and glycine immunoreactivity in cerebellum (Ottersen et al., 1988). Ottersen et al. (1990) also found that GABA and glycine were released from the same terminals in cerebellar Golgi cells. It is well established that the enzyme synthesizing GABA, glutamic acid decarboxylase (EC 4.1.1.15), is specifically localized in the GABA receptor nerve terminals (Fonnum et al., 1970). Due to the lack of specificity of the vesicular GABA and glycine uptake, the key factors in differentiating between GABA and glycine as transmitters in specific terminals seem to be both the synthesis and the high-affinity synaptosomal uptake. It should be kept in mind that the uptake of noradrenaline and dopamine in synaptic vesicles is relatively non-specific, which means that noradrenaline and dopamine are taken up into the same vesicle population (Slotkin et al., 1978).

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